Probing the interactions between iron nutrition, salinity and ultraviolet-B radiation on the physiological responses of wheat (*Triticum aestivum* L.)

A thesis submitted in partial fulfilment of the requirements for the Degree of Master of Applied Science at Lincoln University by H.M. Wong

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Abstract

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By H.M. Wong

When plants are exposed to multiple environmental stress factors, one form of stress can affect the response to another stress. This study used seedlings of a new cultivar of wheat (*Triticum aestivum* L. cv. 1862), grown under factorial combinations of two levels of ultraviolet-B (UV-B) radiation, two salinity regimes and two levels of iron treatment in chelator-buffered nutrient solutions in a growth chamber. A number of morphological and physiological measurements were made. The accumulation of chlorophyll, UV-absorbing compounds and proline in shoots, as well as phytosiderophores (PSs) in root exudates were measured. Feed value measurements included crude protein, water-soluble carbohydrates, acid detergent fibre and Fe in shoots and roots. After 21 days of stress exposure, results showed that Fe deficiency and NaCl stress generally decreased plant growth and function as well as nutritive value, but increased plant biochemical protection traits such as proline accumulation (16.3 fold under salinity stress) and release of PSs (2.4 fold under Fe deficiency). Interestingly, UV-B radiation affected belowground parameters, inducing a 47% reduction in PS release, together with decreasing root DM by 9% and Fe concentration in roots by 7%. When Fe deficiency and NaCl stress were combined, the results showed a decrease in PS release by 3.5 fold compared to unstressed plants. UV-B radiation synergistically increased UV-absorbing compound levels in combination with Fe deficiency, compared to plants grown under optimal Fe levels. This stress combination also resulted in a cumulative effect by decreasing Fe concentration in shoots and roots. However, salt stress did not interact with UV-B radiation for any of the traits measured. In addition, some three-way interactions were noted, with the Fe x NaCl x UV-B stress combination slightly decreasing PS release and resulting in a cumulative effect by decreasing Fe concentration in roots. In conclusion, this study found that aboveground stress factors such as UV-B can affect important aspects of belowground plant function, and that Fe deficiency can interact with UV-B and salinity stress in modifying plant responses to either stress alone.
Keywords: Fe deficiency, NaCl stress, UV-B radiation, wheat, morphology, phytosiderophores, UV-absorbing compounds, proline, photosynthesis, chlorophyll, crude protein, water-soluble carbohydrates and acid detergent fibre.
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<tbody>
<tr>
<td>UV-B</td>
<td>Ultraviolet-B radiation</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>PSs</td>
<td>Phytosiderophores</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>RWC</td>
<td>Relative water content</td>
</tr>
<tr>
<td>WUE</td>
<td>Water use efficiency</td>
</tr>
<tr>
<td>Chl a</td>
<td>Chlorophyll a</td>
</tr>
<tr>
<td>Chl b</td>
<td>Chlorophyll b</td>
</tr>
<tr>
<td>WSC</td>
<td>Water-soluble carbohydrates</td>
</tr>
<tr>
<td>ADF</td>
<td>Acid detergent fibre</td>
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1 General introduction

Wheat, *Triticum aestivum*, is a domesticated grass species that is cultivated worldwide. Globally, wheat is an important human food grain and its production is second only to maize (*Zea mays*) among cereal crops (U.S.D.A., 2002). In the future, wheat farmers throughout the world will face a number of challenges to sustainable production. These challenges include soil salinity, increased ultraviolet (UV) radiation from the depletion of the stratospheric ozone layer, and nutrient deficiencies; all have the potential to reduce wheat production and quality.

Among the elements important for plant growth and development, iron (Fe) plays an important role because of its unique physico-chemical properties. Iron is an essential element for all organisms, is a component of many vital enzymes and required for a wide range of biological functions (Takagi, 1990). However, plants growing on one-third of the world’s soils suffer from Fe deficiency and have associated production losses because of the paucity of readily available soluble Fe (Ma & Nomoto, 1996). The solubility of inorganic Fe is highly dependent on soil pH. Recent research has focused on the effect of Fe in plant roots (Mori, 1999; Murata *et al.*, 2006; Takagi, 1990), but there is little research on the aboveground physiological responses of plants to Fe deficiency.

Soil salinity is an important issue for plant health. Worldwide, approximately 7% of the land area, or 930 million hectares, is negatively affected by elevated concentrations of salts (Sultana *et al.*, 1999; Szabolcs, 1994; Wang *et al.*, 2003). Excess salinity is toxic to plants, affecting plant growth. One of the important impacts of salinity on plants is that it essentially creates a physiological drought in plants (Munns *et al.*, 1995). Furthermore, recent studies have demonstrated that salinity stress reduces the capacity of plants to absorb Fe from alkaline soils (Yousfi *et al.*, 2007).
Stratospheric ozone depletion has led to elevated levels of ultraviolet-B radiation (UV-B, 290-320 nm) on the surface of the Earth (McKenzie et al., 1999). Increased UV-B levels have negative effects on human health, e.g. increased skin cancer levels, (Norval et al., 2006) as well as reducing plant growth and development (Caldwell et al., 2006; Cen & Bornman, 1993). Despite recent measures to reduce the use of ozone depleting substances, mathematical models predict elevated UV-B radiation levels well into the future (Madronich et al., 1995; Zancan et al., 2008). Although the effects of UV-B on plants are well characterised at the physiological level, little is known about the effects of UV-B on underground (root) physiology, particularly in interaction with other environmental factors.

Under experimental conditions, UV-B radiation can lead to either increased sensitivity (Dubé & Bornman, 1992) or decreased sensitivity (Hofmann et al., 2003; Murali & Teramura, 1985) of plants to other stress factors. In the field, plants commonly encounter more than one stress at the same time (for example nutrient stress, drought or salinity and UV-B radiation), but the effects of these combined stresses on plants are still largely unknown. An increasing number of studies have been designed to test the interactions of environmental parameters on plants, such as the interaction between UV-B and water stress (Alexieva et al., 2001; Balakumar et al., 1993; Schmidt et al., 2000; Sullivan & Teramura, 1990; Teramura et al., 1983), interactions between salinity and Fe deficiency (Yousfi et al., 2007) and interaction between UV-B radiation and Fe deficiency (Zancan et al., 2008). However, no that the other is covered of research project has thus far investigated the combined effects of UV-B radiation (an aboveground stress) and salinity (a belowground stress) on plants, including the effects of these combined environmental variables on plant roots. This study provides the first examination of combined effects of UV-B radiation, Fe deficiency and salinity on plants.
Therefore, this thesis reports the effects of interactions between Fe nutrition, salinity and ultraviolet-B radiation on wheat (Figure 1-1). Following a review of the literature (Chapter 2), the experimental design and treatment conditions for these studies are described in Chapter 3. Chapter 4 examines the physiological and morphological effects of Fe deficiency, salinity and UV-B radiation on the stress responsiveness of wheat. In Chapter 5, the effects on biochemistry, especially on selected leaf pigments and amino acids, are investigated to examine possible means that protect plants from stress. Chapter 6 examines how Fe deficiency, salinity and UV-B radiation affect plant nutritive values for protein, water-soluble carbohydrates, acid detergent fibre and Fe concentrations in shoots and roots. Chapter 7 concludes the thesis with a general discussion of the results, written with a view to identifying opportunities for future research resulting from the findings of this work.

The hypotheses of this research project were as follows:

1. Belowground plant attributes will be negatively affected by an aboveground stress factor (UV-B radiation).
2. Aboveground plant attributes will be negatively affected by belowground stress factors (Fe deficiency, salinity).
3. The negative effects of these stress factors applied individually will be altered by the combination of stress factors.
Figure 1-1. Flow diagram of thesis layout.
2 Literature review

2.1 Plants under stress

There are few places in the world where plants are free from exposure to environmental stress. On the contrary, the world is replete with places that experience extreme environmental stress (Taiz & Zeiger, 2006). Therefore, it is important to understand the physiological processes that cause stress injury and the adaptation and acclimatisation mechanisms plants develop in response to environmental stresses. Physiological response to stressors can be divided into two groups (Orcutt & Nilsen, 2000):

1. mechanisms that allow the maintenance of high metabolic activity under mild stress, and reduced activity under severe stress (tolerance mechanisms).

2. reduction of metabolic activity resulting in a dormant state upon exposure to extreme stress (avoidance mechanisms).

The tolerance and avoidance mechanisms of plants to environmental stress result from integrated events occurring at all levels, from the anatomical and morphological to the cellular, biochemical and molecular. This chapter will discuss these effects and the ways in which plants develop tolerance to Fe deficiency, salinity and UV-B radiation stress.
2.2 Wheat

The family *Poaceae* includes all cereals and pasture grasses. Economically, it is the most important group of plants in agriculture (U.S.D.A., 2002). About 87% of New Zealand-grown wheat is used for milling, of which bread and biscuit manufacture are the most important (52%), while 13% is used as forage for livestock (White & Moot, 1999). According to Fowler (2002), wheat has ten major growth stages (Table 2-1). Additionally, most autumn-sown wheat cultivars in New Zealand are essentially “spring” types as they have either weak or no vernalisation requirements, some day length sensitivity and little resistance to cold temperatures (White & Moot, 1999).
Table 2-1. Summary of the growth and development of wheat modified from Fowler (2002).

<table>
<thead>
<tr>
<th>Growth stage of wheat</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Germination</td>
<td>Begins when the wheat kernel uptakes water and the first three seminal roots are produced. After that, the coleotile elongates, pushing the growing point towards the soil surface.</td>
</tr>
<tr>
<td>2. Seedling</td>
<td>The seedling stage starts with the emergence of the first leaf and ends with the appearance of the first tiller. Simultaneously, about six adventitious roots and three leaves support the plant.</td>
</tr>
<tr>
<td>3. Tillering</td>
<td>Crown formation is followed by the emergence of tillers and the development of a secondary or crown root system, which provides nutrients and water during the growing season.</td>
</tr>
<tr>
<td>4. Stem elongation or jointing</td>
<td>This stage starts with the elongation of the internode. The nodes and growing points move upward from the crown to produce a long stiff stem that will carry the head. At the end of this stage, the last (flag) leaf will emerge</td>
</tr>
<tr>
<td>5. Booting</td>
<td>The enlargement of the head and the sheath of the flag leaf occurs. This stage ends when the first awns emerge from the lag leaf sheath and the head starts to force the sheath open.</td>
</tr>
<tr>
<td>6. Heading</td>
<td>This stage extends from the emergence of the tip of the flag leaf sheath until the head has completely emerged, but before flowering.</td>
</tr>
<tr>
<td>7. Flowering</td>
<td>Flowers blossom for pollination and fertilization.</td>
</tr>
<tr>
<td>8. Milk</td>
<td>The milk stage begins with early kernel formation, then the development of the endosperm. Simultaneously, there is a rapid increase in kernel size.</td>
</tr>
<tr>
<td>9. Dough development</td>
<td>The kernel accumulates most of its dry weight during the dough development stage although the kernel still contains 30% water. At the end of this stage, the developing seed is completed with the transport of nutrients from the leaves, stems and spike to the seed.</td>
</tr>
<tr>
<td>10. Ripening</td>
<td>The seed loses moisture in this stage.</td>
</tr>
</tbody>
</table>
2.3 Iron in plants and soils

Iron is an essential element for all organisms (Briat & Lobréaux, 1998). It is required for many vital enzymes, including the cytochromes of the electron transport chain as well as a wide range of other biological functions (Mori, 1999). In plants, Fe is required for metabolic functions that are important in respiration, DNA synthesis, photosynthesis and nitrogen fixation (Sharma, 2006). Iron also catalyzes the single electron reduction of oxygen to form oxidizing free radicals, which may be very damaging to biomolecules. The intracellular concentration of Fe, therefore, requires tight control and so is regulated both at uptake and in storage (Briat & Lobréaux, 1998). Perturbation of Fe homeostasis has severe effects on the physiology and development of plants (discussed in section 2.3.3) (Briat & Lobréaux, 1998). However, Fe acquisition by plants is challenging due to the low solubility of Fe in soil (Guerinot & Yi, 1994).

Although Fe is the fourth most abundant element in the earth’s crust (Mori, 1999), it is not readily available to plants. The most common sources of Fe in soil are the ferric oxides, which are the most stable form of Fe at high pH; therefore, the solubility and dissolution of ferric oxides are important for the availability of Fe to plants (Schwertmann, 1991). In well-aerated soils, at pH > 7, the concentrations of free Fe(II) and Fe(III) in the soil solution are less than $10^{-15}$ M, which is insufficient to meet plant needs (Marschner, 1998). One third of the world’s cultivated soils are calcareous (soil pH > 7.6) and are considered Fe-deficient (Mori, 1999). Thus, Fe deficiency often limits plant growth causing agricultural problems and reduced crop yields (for further discussion see the next section). Plant roots have developed mechanisms that increase iron solubility and availability in the soil solution. These mechanisms are commonly grouped into Strategy I and Strategy II mechanisms (Römheld & Marschner, 1986).
2.3.1 Strategy I plants

Strategy I is an iron acquisition mechanism used by all higher plants except the Poaceae (Römheld et al., 1984). Under Fe-deficient conditions, Strategy I plants increase the solubility of Fe in the rhizosphere to enhance Fe availability to plants in the following ways (Figure 2-1):

1) **H⁺** exudation, which lowers the rhizosphere pH and increases solubility of Fe (Römheld et al., 1984),

2) the release of organic reductants from plant roots that promote the reduction of Fe(III) to Fe(II) (Chaney et al., 1972) and

3) root exudation of Fe-chelating agents (phenolics) that increase the solubility of Fe (Schmidt, 1999).

![Figure 2-1. Diagrammatic representation of plant Fe uptake in Strategy I plants. (PM) is plasma membrane and ® is reductase (Römheld & Marschner, 1986).](image-url)
2.3.2 Strategy II plants

Strategy II plants (the *Poaceae*) produce a special class of Fe-chelator called phytosiderophores (PSs). Phytosiderophores are nonproteinaceous amino acids, such as mugineic acid, that form highly stable complexes with Fe(III) (Takagi *et al*., 1984). These PSs solubilize inorganic Fe(III)-compounds in the soil by chelation (Römheld & Marschner, 1986) and the Fe(III)-PS transport system in the plasma membrane transfers the Fe-chelate into the cytoplasm (Figure 2-2) (Ma & Nomoto, 1996). The process of Fe acquisition by Strategy II plants may be divided into:

1) biosynthesis of PSs inside the roots;

2) secretion of PSs by roots into the rhizosphere;

3) solubilization of sparingly soluble inorganic Fe(III) in soils by chelation with PSs; and

4) uptake of the intact Fe(III)-PS complexes by the roots (Ma & Nomoto, 1996).

Figure 2-2. Model of plant Fe uptake in Strategy II plants: inducible synthesis of phytosiderophores (PSs) from the precursor nicotianamine (NA), extrusion of PSs ⚫ and a special transport system for Fe(III)-PS ⬤ adapted from Römheld & Marschner, 1986.
Chapter 3

Regulation of the phytosiderophore secretion system

During Fe deficiency stress, Strategy II plants increase the production and secretion of ferric chelating agents, PSs, as discussed above, which include 2’-deoxymugineic acid (DMA), mugineic acid (MA) and 3-(epi)hydroxymugineic acid (epi-HMA) (Römheld & Marschner, 1990). However, different Poaceae species excrete different combinations of PSs. For example, maize, wheat, rice and sorghum excrete DMA, whereas barley excretes DMA, MA and epi-HMA (Ma & Nomoto, 1994; Mori, 1999). The diurnal pattern of PS release in the Poaceae was first documented in 1984 in Fe-deficient barley (Takagi et al., 1984). Since then PS release has been documented in over 30 species of Poaceae (Reichman & Parker, 2007). Phytosiderophore release has been found to vary with plant taxa (Gries & Runge, 1992; Yehuda et al., 1996), light period (Nomoto et al., 1987; Reichman & Parker, 2007) and temperature (Ma et al., 2003; Mori, 1999). However, Reichman and Parker (2007) recently undertook the first detailed peer-reviewed research on the regulation of PS secretion and found that PS release was mediated by light not temperature.

In general, secretion of PSs by Poaceae roots occurs in a diurnal rhythm. Secretion starts approximately two hours after sunrise, or after the onset of the light period in growth chamber studies, and quickly reaches a maximum before declining to negligible secretion levels after approximately three hours (Nomoto et al., 1987; Reichman & Parker, 2007). It is not yet known if PS release is controlled by other environmental factors, for example, other regions of the electromagnetic spectrum (e.g. ultraviolet-B radiation) or environmental stresses (e.g. salinity).
The reasons why the Poaceae excrete large diurnal pulses of PSs into the rhizosphere rather than doing this by slow continual excretion are still largely unknown. However, a number of factors potentially affect the efficacy of PS release for solubilising soil Fe:

1) competition by other metal ions for binding sites on the PS molecule (Murakami et al., 1989) and biodegradation of PSs by microorganisms (Takagi et al., 1988);

2) coordination with peak periods of transpiration water flux to transport PSs-solubilised Fe from the rhizosphere soil to the root surface for subsequent uptake and utilization (Parker et al., 2005).

**Uptake of Fe(III)-PS complexes**

Following Fe(III) chelation by PSs, a high-affinity uptake system specific for Fe(III)-PS complexes transports Fe(III)-PS into the epidermal cells of Fe-deficient roots. Yellow-stripe maize (ZmYS1), was the first transporter identified to play a role in the homeostasis transport of various ions including Fe(III), Fe(II), Ni(II), Zn(II), Cu(II), Mn(II) and Cd(II), as their PS complexes (Curie et al., 2001; Schaaf et al., 2004). In addition, ZmYS1 also transports Fe(II)-NA and Ni(II)-NA complexes (Murata et al., 2006). Interestingly, HvYS1 in barley, which is the closest homolog of ZmYS1, with 72.7% amino acid sequence identity and 95.0% similarity, specifically transports Fe(III)-PS complexes (Murata et al., 2006). Recently, it was found that the α-helix structure in the outer membrane loop of HvYS1 is responsible for facilitating selective transportation of the Fe(III)-PS (Harada et al., 2007).
2.3.3 Iron deficiency responses in higher plants

In higher plants, Fe is involved in chloroplast development, harvesting of light energy and transport of electrons from water to NADP$^+$ (Briat & Lobréaux, 1998). Chloroplasts of Fe-deficient plants show a decrease in the concentration of light-harvesting pigments and interveinal yellowing of leaves (Zhang et al., 1991b). Additionally, Fe is involved in electron transport in several components of Photosystem I. The impact of Fe deficiency on the structure and function of the gas exchange parameters, such as photosynthetic rate, stomatal conductance, transpiration rate and WUE was recently assessed in grapevine (Vitis vinifera L. cv. Pinot noir) leaves (Bertamini et al., 2004). Iron deficiency in plants results in a disconnection of the light harvesting complex (LHCI) antenna from Photosystem I even before the Fe-deficient cells develop chlorosis. Severe deficiency of Fe also impairs Photosystem II and the impairment is irreversible, possibly because of structural damage to the Photosystem II reaction centre (Bertamini et al., 2002). As discussed above, Fe is an important co-factor of many enzymes, including those involved in the biosynthetic pathway of chlorophylls (Briat & Lobréaux, 1998). There are several reports of decreases in the protein concentration in the leaves of Fe-deficient plants (Bisht et al., 2002; Yousfi et al., 2007). However, Fe deficiency has been demonstrated to not affect proline content, and thus, Binzel et al. (1987) and Ketchum et al. (1991) suggest that proline does not have a role in protection against Fe deficiency.
2.4 Salinity

Salinity affects about 7% of the world’s land area (930 million ha) and the area of saline land is likely to increase in the future (Szabolcs, 1994). Salinity can result in reduced growth of plants due to an effect that has been described as a “physiological drought” (Taiz & Zeiger, 2006) and also from the direct toxicity of Na\(^+\) and Cl\(^-\) ions. Physiological drought means a shortage of water within the plant even when growing under moist but saline soil conditions (i.e. high concentration of NaCl), or in saline culture solutions.

2.4.1 Damaging effects of salinity in plants

Generally, many major crops are salt-sensitive and will not complete a full life cycle above 100 mM NaCl (Taiz & Zeiger, 2006). However, plants can be divided into two broad groups on the basis of their response to high concentrations of salts. The halophytes, or salt-tolerant plants, can grow for some time in relatively high concentrations of salt without visible injury, although at reduced rates (Parida & Das, 2005). For example, barley can grow for more than four weeks in a 100 mM NaCl solution with little visible injury (Yousfi et al., 2007). Glycophytes, or salt-sensitive plants, experience toxicity symptoms at relatively low salinity levels (Parida & Das, 2005). For example, in lupin (Lupinus albus L.), salt quickly accumulates in the leaves, causing injury and abscission within a few days of exposure to 100 mM NaCl (Munns, 1988).

Plant growth in response to salinity has two phases (Munns et al., 1995). In the first phase there is a large decrease in growth rate caused by the salt outside the roots (osmotic response). The osmotic response is similar to drought stress and results in decreased growth, stomatal closure (causing lower CO\(_2\) availability), reduced photosynthetic activity and a decrease in cell water potential (Bajji et al., 2001; Chaves, 1991; Jefferies, 1994; Ranjbarfordoei et al., 2000). In the second phase, there is an additional decline in growth caused by salt building up to toxic levels within the plants (salt-specific response) (Munns et al., 1995). Plants grown under toxic levels of salinity will display symptoms of chlorosis and necrosis on the edge of their leaf blades (Munns et al., 1995). Additionally,
salinity stress will induce the production of reactive oxygen species (ROS), which are potent oxidants that can lead to cell death because of lipid peroxidation (membrane destruction), protein oxidation, enzyme inactivation and RNA/DNA damage (Taiz & Zeiger, 2006). For example, under salinity stress plants increase photorespiration and mitochondrial respiration when electron flow is too great for the normal electron acceptors of metabolism (Taiz & Zeiger, 2006).

2.4.2 Protective responses of plants to salinity

2.4.2.1 Osmotic adjustment

Osmotic adjustment is the primary acclimitization response of plants to water deficiency or salinity (Nepomuceno et al., 1998) because of increased water retention following the accumulation of osmolytes. Osmotic adjustment can be defined as net solute accumulation in plant cells during the development of a water deficiency, exclusive of the effects of decreasing leaf water content (Morgan, 1983) When plants are exposed to an osmotic stress, such as salinity, the resulting decrease in plant water potential frequently results in a decrease in the solute potential of plant cells. In the initial stress phase this is often a passive process due to the water being lost from the cell. However, in osmotically adjusting plants this is followed by an active cellular accumulation of organic solutes (also termed “compatible solutes”, e.g. glycinebetaine, sorbitol and proline) that do not interfere with enzyme functioning (Taiz & Zeiger, 2002). This process of osmotic adjustment, also known as osmoregulation, enables cells to maintain their pressure potential when subjected to osmotic stress.

In the field, under conditions of saline soil water or irrigation, plants can continue to absorb water only as long as their water potential is lower (more negative) than that of the soil water. Under salinity the soil water potential is reduced, necessitating a reduction of plant water potential to ensure continued water uptake. Thus, osmotic adjustment is a process by which plant water potential can be decreased without an accompanying decrease in turgor pressure or decrease in cell volume (see section 2.4.1).
2.4.2.1.1 Proline

Generally, proline accumulates under conditions of water stress, and so, proline accumulates during the osmotic phase of the salinity response in plants (Taiz & Zeiger, 2006). In response to salinity stress, proline accumulates in the cytosol while Na\(^+\) and Cl\(^-\) are sequestered in vacuoles to adjust the osmotic balance between cytosol and vacuole (Kemble and Mac Pherson, 1954). In addition to its unique function in osmotolerance, proline also plays an important role in morphogenesis as a major constituent of cell wall structural proteins in plants (Taiz & Zeiger, 2002). However, proline can also accumulate in a range of plants in response to a wide range of environmental stresses (Table 2-2). Other suggested roles for proline in the stress response of plants are the scavenging of free radical reactive oxygen species (ROS) (Smirnoff & Cumbes, 1989) and metabolic signalling (Girousse et al., 1996; Shetty et al., 2002).

Table 2-2. Summary of environmental stimuli capable of inducing proline accumulation in plants adapted from Hare & Cress (1997).

<table>
<thead>
<tr>
<th>Stress</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water deficit</td>
<td>Kemble &amp; Mac Pherson (1954)</td>
</tr>
<tr>
<td></td>
<td>Delauney &amp; Verma (1993)</td>
</tr>
<tr>
<td></td>
<td>Heuer (1994)</td>
</tr>
<tr>
<td></td>
<td>Bajji et al. (2001)</td>
</tr>
<tr>
<td>High salinity</td>
<td>Delauney &amp; Verma (1993)</td>
</tr>
<tr>
<td></td>
<td>Heuer (1994)</td>
</tr>
<tr>
<td>Nutrient deficiency</td>
<td>Vaucheret et al. (1992)</td>
</tr>
<tr>
<td>Enhanced UV-irradiation</td>
<td>Pardha et al. (1995)</td>
</tr>
</tbody>
</table>
2.4.2.2 Ion exclusion

Plants can avoid salt injury by excluding excess ions from leaves and/or the compartmentalisation of ions in vacuoles. Ion exclusion mainly controls NaCl uptake into roots and movement within the plant. For example, radial transport of Na\(^+\) and Cl\(^-\) from the soil solution into roots can be regulated in epidermal and cortical cells before reaching the xylem (Taiz & Zeiger, 2006). Additionally, plants can absorb Na\(^+\) from the transpiration stream (xylem sap) or exclude salt from their meristems (Taiz & Zeiger, 2006). However, some halophytes, such as salt cedar (Tamarix sp.) and salt bush (Atriplex sp.), do not exclude ions at the root, but instead have salt glands or bladders at the surface of leaves that act as a sink (Taiz & Zeiger, 2006).

2.4.2.3 Stomatal conductance

When salinity stress occurs rapidly or the plant reaches its full leaf area before the initiation of stress, stomatal closure protects the plant against immediate physiological drought by reducing evaporation from the existing leaf area (Delfine et al., 1998; Jiang et al., 2006). Uptake and loss of water in guard cells changes their turgor pressure and modulates stomatal opening and closing. During salinity exposure, the solute potential around the stomatal guard cells is strongly reduced, resulting in water diffusion from the guard cells and, as a consequence, stomatal closure to maintain cell turgor and reduce evaporation loss (Shope et al., 2003). Thus, stomatal closure can be seen as an important water retention mechanism in plants exposed to salinity stress (Delfine et al., 1998; Jiang et al., 2006).
2.4.2.4 Cell growth responses

The osmotic response phase under salinity stress acts like a physiological drought and thus results in the water content of tissues and cells being reduced (Taiz & Zeiger, 2002). Plants frequently respond to the physiological water deficits by partially or completely closing stomata to maintain cell turgor (Taiz & Zeiger, 2006). Cell growth is one of the most sensitive parameters under water deficit stress due to its dependence on turgor. Reduced cell growth results in reduced leaf area, and therefore, the first measurable sign of salinity stress is often smaller leaves due to decreased leaf elongation (Hu & Schmidhalter, 1998). A reduction in leaf area affects yield and photosynthesis, as a result of reduced potential to capture light.

Typically, as the water content of the plant decreases, it results in cells shrinking and the cell walls relaxing (Chaves et al., 2003b). This decrease in cell volume is associated with lower turgor pressure and the subsequent concentration of solutes in the cells (Taiz & Zeiger, 2002). As a result, plasma membranes become thicker and more compressed because they cover a smaller surface area than previously (Fu, 2003; Thomas et al., 1999).
2.5 Ozone depletion and UV-B

2.5.1 Ozone depletion and increased UV-B

Ozone (O$_3$) is an unstable, pungent, toxic form of the element oxygen with three atoms in its molecule. Ozone is formed from oxygen naturally in electrical discharges or by UV radiation (Taiz & Zeiger, 2006). The “ozone layer” is a high concentration of ozone that forms in a layer in the stratosphere at an altitude of about 20-40 km above the Earth (Taiz & Zeiger, 2006). Ultraviolet radiation emitted from the sun is a continuous spectrum, arbitrarily divided into three wavebands, UV-C (200-280 nm), UV-B (280-315 nm) and UV-A (315-400 nm) (Madronich et al., 1998). The UV-C is completely absorbed by the earth’s atmosphere, while UV-B and UV-A can reach the surface of the earth. The ozone layer absorbs about 90% of the UV-B radiation reaching the Earth from the sun (WMO, 1995). The amount of UV-B radiation reaching the surface of the Earth is highly variable as it is influenced by many factors including ozone concentration in the stratosphere, sun angle, cloud cover, aerosol extinctions, albedo, day length and altitude (Hunt & McNeil, 1998). These factors tend to result in UV-B irradiance being highest during the summer at lower latitudes and higher altitudes (Madronich et al., 1995). Additionally, UV radiation is more intense in the southern hemisphere because of hemispheric differences in ozone and also because the closest earth-sun separation occurs during the Southern Hemisphere summer (McKenzie et al., 1999).

The natural UV-B distribution at the Earth’s surface has been significantly affected by anthropogenic activity, primarily through the release of man-made chlorine and bromine compounds (chlorofluorocarbons, CFCs) that diffuse into the stratosphere and destroy ozone (WMO, 1995). The greatest ozone depletion occurs in the southern spring at the highest latitudes. At the highest latitudes, when sunlight returns to the very cold stratospheric clouds in the polar vortices after winter it provides ideal conditions for chlorine and bromine to be rapidly catalysed, causing ozone destruction (SORG, 1996). In the mid 1980s, scientists discovered that a “hole” developed periodically in the stratosphere above Antarctica. It was found that the ozone layer there was thinned by as much as 40-50% from its normal concentrations (Madronich et al., 1995). Plants are
naturally exposed to UV-B radiation from the electromagnetic solar spectrum. In addition, as a result of anthropogenic ozone depletion and the associated increase in UV-B radiation at the Earth’s surface, plants may experience elevated UV-B exposure. These increases in exposure to UV-B radiation can lead to changes in plant physiology, including plant growth, morphological change (Cen & Bornman, 1993; Wilson & Greenberg, 1993) and photosynthetic damage (Allen et al., 1998), as well as the accumulation of phenolic compounds (Rozema et al., 1997; Soheila & Mackerness, 2000).

2.5.2 Damaging effects of UV-B radiation on plant physiology

Generally, leaves absorb over 90% of incident UV-B radiation (Cen & Bornman, 1993). Leaf surface reflectance in this wavelength range is thus generally below 10% and there is negligible transmission of UV-B through leaves (Cen & Bornman, 1993). Ultraviolet-B radiation that is absorbed may induce morphological and physiological changes in plants.

2.5.2.1 Plant growth and morphology

Ultraviolet-B radiation induces a range of morphological effects in plants including leaf thickening, cotyledon curling, inhibition of hypocotyls, elongation stem and leaf elongation, axillary branching and shifts in the root-shoot ratio (Cen & Bornman, 1993; Wilson & Greenberg, 1993). The effects of UV-B radiation on reproductive morphology include increased flower numbers (Day et al., 1993) or flower diameter (Petropoulou et al., 1995). Some of these responses constitute a stimulation of the growth of a specific tissue (e.g. axillary branching, leaf thickening); while others arise from an inhibition of plant growth (e.g. diminished hypocotyl elongation). However, not all plant species respond in the same way to UV exposure. Following growth under high UV-B levels, shoot height decreased in *Triticum aestivum* and *Avena sativa*, but increased in *Amaranthus retroflexus* and *Kochia scoparia* (Barnes et al., 1990). Furthermore, all four species showed increased branching (tillering or axillary branching) in response to UV-B (Barnes et al., 1990). Similarly, UV-B radiation induced an increase in the root-shoot ratio in sweetgum (Sullivan et al., 1994) but a decrease in cassava (Ziska et al., 1993) under field conditions. Barnes et al. (1990) suggested that monocotyledonous plants are
more morphologically adapted to UV-B than dicotyledonous plants. This is thought to be due to a variety of morphological and physiological features in grasses and other monocots. Compared to dicots, this includes more vertical leaf arrangements (thus reducing the incidence of direct damage from UV-B radiation), the presence of alkaloids that can have UV-screening properties, a more diverse distribution of the photorepair enzymes and a well-shielded meristematic region in the protective basal sheath (Cybulski & Peterjohn, 1999; He et al., 1993; Musil & Wand, 1999; Van & Garrard, 1976).

2.5.2.2 Photosynthetic damage

One of the reported responses of plants to UV-B radiation is a reduction in the rate of photosynthesis particularly under high levels of UV-B radiation (Allen et al., 1998; Bogenrieder & Douté, 1982; Sullivan et al., 1994; Teramura et al., 1983). Ultraviolet-B radiation can potentially impair the performance of all the main component processes of photosynthesis, i.e., photophosphorylation reactions of the thylakoid membrane, the CO2-fixation reactions of the Calvin cycle and stomatal control of CO2 supply (Figure 2-3) (Allen et al., 1998). One effect on photosynthesis that is often reported is that the Photosystem II (PSII) reaction centre (D1) increases production of active toxic oxygen species in response to UV-B. This results in the inactivation and disruption of the photophosphorylation reactions of the thylakoid membrane in photosynthesis (Allen et al., 1998). It also causes reduction in chlorophyll content, possibly by reduced expression of genes encoding for chlorophyll binding proteins (Teramura et al., 1983). In the Calvin cycle, UV-B radiation can result in a decline in rubisco activity because of the deactivation or loss of the enzyme, RuBP carboxylase (Quick et al., 1991). Ultraviolet-B-induced inhibition of another component of photosynthesis may result in stomatal closure in response to the reduced demand for CO2 (Teramura et al., 1983). It has to be pointed out that most of these effects of UV-B on photosynthesis are found in laboratory studies where very high levels of UV-B radiation are applied, together with low levels of photosynthetically active radiation (PAR) (Searles et al., 2001).
Figure 2-3. A schematic representation of the main processes in C₃ photosynthesis in higher plants, of photophosphorylation, Calvin cycle and stomatal conductance. (A) is CO₂ assimilation, (Cᵢ) is intercellular CO₂ concentration and (Cₐ) is extracellular CO₂ concentration (Allen et al., 1998).

2.5.3 Protective physiological responses to UV-B radiation

In response to the damaging effects of UV radiation, plants have developed defence mechanisms against UV-B while allowing photosynthetically active radiation (PAR) to penetrate through the outer cell layers to support photosynthesis in the mesophyll and palisade tissues (Cen & Bornman, 1993). This may involve the rapid biosynthesis of protective pigments that absorb the damaging UV radiation (Jordan, 1996). There are also a series of enzymes called photolyases that are capable of repairing critical molecules, such as DNA (Cen & Bornman, 1993).
2.5.3.1 Morphological changes

Generally, changes in plant architecture combine to diminish the exposure of cells or leaves to UV-B radiation (Barnes et al., 1996). Morphological changes caused by UV-B radiation can also contribute to protection against UV-B (Cen & Bornman, 1993). For example, an increase in surface wax, leaf hairs and leaf bladders can reflect UV-B radiation from epidermal and cuticular structures (Cen & Bornman, 1993). Additionally, inhibition of hypocotyl elongation has been hypothesized to minimize UV-B exposure of the emerging tomato seedling until it has accumulated UV-screening pigments (Ballaré et al., 1995). It has been suggested that the UV-B radiation response of leaf thickening diminishes the number of cell layers exposed to ambient UV-B radiation, since UV-B radiation penetrates only the upper cell layers of a leaf (Cen & Bornman, 1993). Similarly, the reduction of apical dominance (decreased shoot length and increased axillary branching) will, within a canopy, diminish exposure to direct sunlight (including its UV-B component) as leaves of short, bushy plants are more likely to be shaded (Barnes et al., 1996). Additionally, UV-B induces plant morphological changes such as the development of short (stunted) stems and decreased leaf area, which could affect photosynthetic capacity and decrease plant productivity in the form of biomass accumulation (Cen & Bornman, 1993).

2.5.3.2 Accumulation of phenolic compounds

Plants are protected against the penetration of UV-B into internal tissues by accumulating phenolic compounds to absorb the excess UV-B radiation (Rozema et al., 1997; Soheila & Mackerness, 2000). The most common classes of phenolic compound are the flavonoids, produced by the phenylpropanoid pathway. Flavonoids are water soluble flavone and flavonol glycosides or their derivatives, particularly kaempferol and quercetin, and are located in vacuoles (Rice-Evans et al., 1997). Examples of flavonoids are produced primarily in the epidermal layers of the leaves and absorb UV-B radiation effectively while transmitting PAR to the chloroplasts (Jordan, 1996). In addition to their role as sunscreens, flavonoids are also known to have an antioxidant function and can help dissipate UV-B radiation within the leaf (Hofmann, 2000).
2.6 Interactions between salinity, UV-B and iron stresses in plants

Under field conditions, where environmental factors tend to co-occur and plants commonly experience multiple stresses simultaneously, the net effect of two or more concomitant stresses on a given physiological or morphological growth characteristic could be manifested in several different responses. First, the response to one stress may help alleviate the response to another stress (Caldwell et al., 2006). In this compensatory (antagonistic) case, a plant receiving two simultaneous stresses would be less affected than when only receiving a single stress. Secondly, the combined response may be the sum of the responses to each individual stress. That is, the net effect of two simultaneous stresses could also be additive. A synergistic interaction could occur in cases where one stress increases the impact to another stress. From reviewing the literature, there is some support for multiple stresses having positive compensatory effects with plants experiencing several stresses concomitantly being protected against the occurrence of an additional stress (Teramura et al., 1983; Zancan et al., 2006). This can be explained by the conferring of cross-tolerance of one stress to another stress. For example, the UV-B-induced closing of stomata could protect plants against desiccation under saline stress conditions. Several studies have been expressly designed to test the effects of the interaction of two of the three environmental factors used in this study, including UV-B radiation and salinity stress (Fedina et al., 2003; Çarkılar et al., 2008), UV-B radiation and iron deficiency (Zancan et al., 2008) and salinity and iron deficiency (Yousfi et al., 2007, Rabhi et al., 2007). However, no research has been conducted on the three-way interaction between Fe deficiency, NaCl stress and UV-B radiation effects on plants. It can be hypothesised that the combination of stress factors will result in cross-tolerance mechanisms in plants, compared to the effects of one of these stress factors alone. Additionally, there is little research into the effects of UV-B radiation on roots. Thus, the research in this thesis combined a shoot-based stress (UV-B radiation) with two root-based stresses (salinity and Fe deficiency) and took measurements of both shoot and root responses to these multiple stresses.
3 General Materials and Methods

Experimental design
This experiment was conducted in a large walk-in growth chamber (Conviron, PGV36, Canada) at Lincoln University, Canterbury, New Zealand. The experiment was laid out as a randomised complete block within environments design (Figure 3-1), with two levels of UV-B radiation (1 kJ m$^{-2}$ d$^{-1}$ and 15 kJ m$^{-2}$ d$^{-1}$ biologically effective UV-B), two salinity regimes (no added-NaCl and 75 mM of NaCl) and two concentration levels of iron (Fe) treatment (75 µM of Fe (pFe = 16.5) and 3.75 µM of Fe (pFe = 17.8)). Wheat plants were grown in six replicated pots per treatment giving a total of 48 pots for the whole experiment. The two levels of UV-B radiation were applied by dividing the growth chamber in half with a UV absorbing screen. An electric fan placed under one side of the tables was used to circulate air and maintain equivalent environmental conditions in the two half-chambers. Ultraviolet and visible light sensors were placed in the middle of the tables between the two blocks of the high UV-B radiation.

Every four days throughout the experiment the pots were re-randomized within each block and the blocks were cycled progressively clockwise through the growth chamber at each re-randomization (Figure 3-1). In addition, for the UV-treatment, the corresponding plants were switched between the alternate halves of the growth chamber at each re-randomization, to further ensure all plants were exposed to comparable room conditions (see below and Figure 3-1).
Plant materials and growth conditions

A new wheat cultivar (*Triticum aestivum* cv. 1862) provided by Luisetti Seeds Ltd (Canterbury, New Zealand), was used as the test species. Seeds were surface-sterilized in 0.4 M sodium chloride solution for 10 min and then rolled in germination towels moistened with deionised water. The seeds were placed in a growth chamber with 24 h light and a 12 h:12 h, 20°C:15°C temperature regime. After six days, seedlings with a height of approximately 4 cm were transferred to 4 L containers filled with nutrient solution (Day 0) in the same growth chamber. The light regime was then changed to 12 h light: 12 h dark the 20°C:15°C temperature regime was maintained and the relative humidity was set at 65%. Seedlings were placed inside plastic tubes and fitted into small holes in the lid of each container with the roots held securely in place using small squares of foam (Figure 3-2). Fifteen seedlings were planted per pot and one hole in the lid was used for aeration. The solution cultures were aerated using air stones (Kordon, Novalek Inc, Hayward, CA, USA) linked to silicon tubing aeration lines, connected to an aquarium pump, similar to that employed by Reichman and Parker (2007) (Figure 3-3).
Figure 3-2. Seedling planted in a plastic tube and fitted through small holes in the ice cream lid.

Figure 3-3. Photograph of the experimental set-up.
Chapter 3

Solution culture
A chelator-buffered nutrient solution was used to buffer Fe and trace-metal activities throughout the experiment (Reichman & Parker, 2007). Basal nutrients were supplied as (µM): NO₃⁻, 4750; NH₄⁺, 250; P, 80; K, 1080; Ca, 2000; Mg, 500; Mn, 0.6; Zn, 8; Cu, 2; B, 10, Mo, 0.1; Ni, 0.1; and Cl, 21.4 (Pedler et al., 2000). To control trace-metal activity the solutions contained 35.7 µM hydroxyethylethylenediaminetriacetic acid (HEDTA), representing a 25 µM excess above the sum of the Mn, Cu, Zn and Ni concentrations. On days 0 to 7, all seedlings were grown in complete solution culture that included 75 µM Fe as FeCl₃ plus another 75 µM HEDTA to buffer the Fe activity. To buffer the solution culture at pH 6, the solution contained 1mM 2-(N-morpholino)ethanesulfonic acid (MES) buffering agent and 0.5 mM NaOH. Daily measurements of pH were made and the pH was adjusted, as necessary, with 0.1 M NaOH or HCl. The amount of NaOH and HCl used was kept at a minimum so as to not affect the salinity stress treatment. The solution culture was changed on days 8, 15 and 18 after the seedlings were transplanted.

Experimental treatments
The three treatments commenced on day 8. The chemical speciation program GEOCHEM-PC was used to calculate the pFe of each treatment (Parker et al., 1995) where the pFe of a solution is defined as –log₁₀ (Fe³⁺ activity). In the control Fe treatment (pFe = 16.5), Fe was supplied as 75 µM Fe as FeCl₃ (plus 75 µM HEDTA to buffer the Fe activity). In the Fe-deficient treatment (pFe = 17.8), Fe was supplied as 3.75 µM FeCl₃ (plus 3.75 µM HEDTA to buffer the Fe activity). Two levels of salinity were applied: a control treatment of 0 mM NaCl and a moderate salinity treatment of 75 mM NaCl (determined during a pilot experiment, data not provided). There were two UV-B radiation levels, one on each side of the screen in the centre of the growth chamber (Figure 3-3), a control treatment of 1 kJ m⁻² d⁻¹ biologically effective UV-B and a UV-B supplementation treatment with 15 kJ m⁻² d⁻¹ biologically effective UV-B, normalised to 300 nm (Caldwell, 1971). The UV-B supplementation treatment represented a level of approximately 24% summer ozone depletion above Canterbury, New Zealand (NIWA, 2007). Photosynthetic photon flux (PPF) was 330-350 µmol m⁻² s⁻¹. UV-B and light levels were gradually increased during the first hour of the light period, then gradually decreased during the final hour.
**Harvest activities**

On days 19 and 20, photosynthesis rate, stomatal conductance and transpiration rate were measured using a LI-COR 6400 gas exchange system (LI-COR Biosciences, Lincoln, Nebraska). Gas exchange parameters were measured on two days; replicates 1 to 3 were measured on day 19 and replicates 4 to 6 on day 20. The purpose of this was to make sure all plants were measured between 3 and 4 h after the light period commenced, as stomatal opening is likely to be optimal during this period (Hunt & McNeil, 1998).

On day 21, root exudate samples for phytosiderophore (PS) analysis were collected between 2 to 5 h after the light period commenced. This time period was chosen because PS release has been determined to be at its optimum in wheat during this time period (Reichman & Parker, 2007).

At the end of the experimental period (day 22), leaf area and fresh weight (shoots and roots) were measured. Shoots and roots were cleaned by washing in 2% detergent (Decon Labs Ltd, East Sussex, England) in reverse osmosis (RO) water and then washing three times in RO water. For further analyses and storage, plant samples were freeze-dried for 2.5 weeks (Cuddon Ltd Freezer Dryer, Blenheim, Canterbury) and then ground in a mortar with liquid nitrogen. Samples were subsequently stored at -35°C until dry weight, chlorophyll, UV-B absorbing compounds, proline, crude protein, water-soluble carbohydrate (WSC), acid detergent fibre (ADF) and Fe were measured in the shoots and roots.
Statistical analyses

The experiment, which used a randomized complete block within environments design, was analysed with the statistical computer package GENSTAT (Genstat, 2005) to compare treatment effects. It should be noted that due to the practical constraints of using UV-B supplementation, one growth chamber with one partitioning of UV-B treatments was used. Thus there was no replication of the UV treatment, and any test for a UV main effect is based on the variation within each UV environment. Thus, the UV treatment is confounded with the two main blocks. The analysis, with GENSTAT (restricted maximum likelihood) REML, had UV, NaCl and Fe as fixed (treatment) effects and blocks within UV as a random effect. The analysis was repeated, with UV and blocks within UV as a random term, and NaCl and Fe deficiency and their interaction as fixed terms, as well as UV by NaCl and UV by Fe. Data that passed homogeneity of variance and normal distribution of error \((P<0.05)\) were tested untransformed using GENSTAT REML. Data that failed homogeneity of variance and normal distribution of error testing \((P>0.05)\) were square root or \(\log_{10}\) transformed to achieve homogeneity in the variance of residuals, and statistical significance was based on analysis of the transformed data. Differences were considered significant at \(P<0.05\), and marginally significant up to \(P<0.10\). It also needs to be pointed out that due to the large number of possible interactions in this study, there are 16 interaction significance tests for each parameter, increasing the chance of actual non-significance for a significant result at \(P < 0.05\). The least significant differences (5% level) of REML were used to further separate means. The output of all analyses is available in the appendices on the CD included with this thesis. Results in this thesis are first presented as main effects, i.e. Fe deficiency, NaCl and UV-B, followed by presentation of two-way and three-way interactions of these factors. In each chapter, the statistical significance levels from the corresponding REML analyses are fully tabulated, and the description of results in the text refer to these tables. Raw and transformed data used for these analyses are listed in the appendices.
Chapter 4

4  Effects of induced stress on aspects of the morphology and physiology

4.1 Introduction

Numerous morphological and physiological changes in plants can be used as indicators of responses to environmental stress, including those of growth and photosynthetic gas exchange. As noted earlier (Chapter 2), there has been little research on the interaction of Fe deficiency and other stress factors on gas exchange in plants. Recent findings point to changes in root morphology and root to shoot ratio in response to Fe deficiency stress (Crowley et al., 2002; Marschner, 1998; Yousfi et al., 2007). Under saline conditions, reduction in growth can be one consequence of a number of plant physiological responses, including modification of leaf surface expansion, water status, stomatal function and photosynthetic efficiency (Munns, 1993). Exposure to UV-B radiation can lead to morphological change and physiological damage in plants (Cen & Bornman, 1993). The response of plants to changes in UV-B radiation may also depend upon other concomitant stresses (Caldwell et al., 2006). There are also some indications that enhanced UV-B radiation may be beneficial to some plants in alleviating the adverse effects of drought and nutrient limitations (Musil & Wand, 1994; Petropoulou et al., 1995). In addition, research has shown cross tolerance effects of UV-B radiation interacting with drought stress (Hofmann et al., 2003; Murali & Teramura, 1985). However, there has been no research focussed on the interaction of multiple stress factors on plants.

The aim of this study was to investigate how three stress factors (Fe, NaCl and UV-B radiation) affect physiological and morphological attributes of wheat, and how the individual effects might change when these stress factors are applied at the same time. The objective of this chapter is to determine the aboveground and belowground responses of plants to Fe, NaCl and UV-B exposure, as well as to a combination of these stress factors.
4.2 Materials and methods

Plant growing conditions and the experimental design were outlined earlier in Chapter 3. Additional methods and details of the analyses of plant morphological and physiological changes are described below.

**Plant dry weight**

Plants were harvested and their dry weights determined, as outlined earlier (Chapter 3).

**Root to shoot ratio**

Root:shoot ratio was calculated using the root dry weight divided by the shoot dry weight.

**Leaf area**

The total fresh leaf area of each plant was measured using a LI-COR (model 3100) area meter (LI-COR Biosciences, Lincoln, Nebraska), as outlined earlier (Chapter 3).

**Relative water content**

The first fully expanded distal leaf from each plant was harvested and weighed to determine the fresh mass (FM). The leaf was then incubated in RO water for 24 h at 20°C in high light, to allow full hydration. The mass of the water-saturated leaves (TM) was measured and the leaf subsequently dried at 80°C for 48 h and re-weighed to determine the dry mass (DM). The relative water content (RWC) was calculated using the following equation (Bajji *et al.*, 2001):

\[
\text{RWC} = \left( \frac{\text{FM}-\text{DM}}{\text{TM}-\text{DM}} \right) \times 100
\]
Gas exchange

The first fully expanded leaf was selected from each pot to measure gas exchange parameters \textit{in situ}, using a LI-COR model 6400 infrared gas analyzer (IRGA) (LI-COR Biosciences, Lincoln, Nebraska). The parameters measured included leaf photosynthesis rate, stomatal conductance and leaf transpiration rates. Each leaf lamina was enclosed in the LI-COR 6400 chamber for ten minutes to ensure equilibrium was reached before recording the result. Stomatal opening is at a likely optimum between approximately three and five hours after the commencement of the light period (Hunt & McNeil, 1998), but for logistical reasons not all pots could be measured on the same day. Consequently, half of the pots were measured on day 19 (replicates 1-3) and the remaining half on day 20 (replicates 4-6). On both days, measurements were made between three and four hours after the beginning of the light period (Hunt & McNeil, 1998). Leaf physiological water use efficiency (WUE) for individual leaves was calculated as the photosynthesis rate divided by the transpiration rate (Stępień & Kłobus, 2006).
4.3 Results

4.3.1 Plant growth and morphology

4.3.1.1 Visual

Wheat plants grown under low UV-B radiation, sufficient Fe and no NaCl stress conditions (Figure 4-1; Figure 4-2a) flourished and appeared very healthy. The Fe deficiency treatment generated chlorosis on the young leaves (Figure 4-1; Figure 4-2c and d). Visual NaCl stress symptoms were observed as reduced plant growth and the leaf tips of old leaves turned pale yellow with obvious leaf senescence (Figure 4-1; Figure 4-2b and d). There were no visual differences in the plants treated with the low or high UV-B irradiation (Figure 4-1; Figure 4-2).

4.3.1.2 Main effects

4.3.1.2.1 Iron

The Fe deficiency treatment reduced shoot dry matter (DM) by 65%, root DM by 59% and leaf area by 65% compared to the Fe sufficient treatment (Figure 4-3a, b and e; Table 4-1). The root:shoot increased by 16% in the Fe-deficient plants but the RWC was not affected by Fe deficiency (Figure 4-3c and d; Table 4-1).

4.3.1.2.2 Salt

The NaCl treatment showed reduced shoot DM by 50%, root DW by 32% and leaf area by 59%, while the root:shoot increased by 34% compared to the no NaCl treatment (Figure 4-3a, b, and e; Table 4-1). Salt stress reduced the RWC in the leaves of the wheat plants by 5% compared to the no NaCl treatment (Figure 4-3d; Table 4-1).
Figure 4-1. Wheat (*Triticum aestivum*) planted in solution culture under different Fe, NaCl and UV-B levels.
Figure 4-2. Growth response of wheat (*Triticum aestivum*) planted in solution culture under different combinations of Fe nutrition and NaCl stress. (a) +UV-B x +Fe x -NaCl, (b) +UV-B x +Fe x +NaCl, (c) +UV-B x –Fe x –NaCl and (d) +UV-B x –Fe x +NaCl.
4.3.1.3

Figure 4-3. Shoot dry matter (DM) (a), root dry matter (DM) (b), root:shoot (c), relative water content (RWC) (d) and leaf area (e) of wheat plants grown in solution culture under different Fe, NaCl, and UV-B levels. Values are mean ± standard error (n = 6), except RWC is n = 3.
Table 4-1. Summary of significance levels from REML analyses describing the effects of Fe deficiency, NaCl and UV-B radiation on growth and morphology of wheat plants. DM, dry matter and RWC, relative water content.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Shoot DM (g plant(^{-1}))</th>
<th>Root DM (g plant(^{-1}))</th>
<th>Root:shoot</th>
<th>Leaf area (cm(^2) plant(^{-1}))</th>
<th>RWC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe (***)</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>NS</td>
</tr>
<tr>
<td>NaCl (***)</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>**</td>
</tr>
<tr>
<td>UV-B (NS)</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Fe x NaCl (***)</td>
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<td>***</td>
<td>NS</td>
<td>***</td>
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</tr>
<tr>
<td>Fe x UV-B (NS)</td>
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<td>NS</td>
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<tr>
<td>NaCl x UV-B (NS)</td>
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<td>NS</td>
<td>NS</td>
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<tr>
<td>Fe x NaCl x UV-B (NS)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
</tr>
</tbody>
</table>

Raw data are shown in Appendix 4-1. Significance levels are: \(***\) \(P < 0.001\); \(**\) \(P < 0.01\); \(*\) \(P < 0.05\); NS, \(P \geq 0.10\).

4.3.1.3.1 \textit{UV-B}

Exposure of wheat plants to UV-B radiation did not significantly alter the shoot DM, RWC or leaf area (Figure 4-3a, d, and e; Table 4-1). However, UV-B radiation resulted in a small but significant decrease in the root DM and root:shoot of 9% and 5%, respectively (Figure 4-3b and c; Table 4-1).

4.3.1.4 Interaction effects

4.3.1.4.1 \textit{Fe x Salt}

When no NaCl stress was applied, the Fe deficiency treatment decreased the shoot DM by 70%, root DM by 65% and leaf area by 70% (Figure 4-3a, b, and e; Table 4-1). Under NaCl stress conditions, the Fe deficiency treatment decreased shoot DM by 54%, root DM by 48% and leaf area by 49% (Figure 4-3a, b, and e; Table 4-1). Under Fe sufficient conditions, application of NaCl decreased shoot DM by 56%, root DM by 39% and leaf area by 65% (Figure 4-3a, b, and e; Table 4-1). When Fe deficiency conditions were present, the additional NaCl stress decreased shoot DM by 32%, root DM by 10% and leaf area by 40% (Figure 4-3a, b, and e; Table 4-1). There was no interaction between Fe deficiency and NaCl stress on the root:shoot or RWC (Figure 4-3c and d; Table 4-1).
4.3.1.4.2 Other stress interactions

No effects on plant growth and morphology were found for the other two-way (Fe x UV-B stress and NaCl x UV-B stress) or three-way (Fe x NaCl x UV-B stress) interactions (Figure 4-3; Table 4-1).

4.3.2 Leaf gas exchange

4.3.2.1 Main effects

4.3.2.1.1 Iron

Photosynthetic gas exchange was reduced by Fe deficiency by 66%, stomatal conductance by 32%, transpiration by 21% and water use efficiency by 40% (Figure 4-4a, b, and c; Table 4-2).

4.3.2.1.2 Salt

Salinity stress increased physiological WUE by 23% (Figure 4-4d; Table 4-2), in parallel with a marginally significant increase in photosynthesis ($P = 0.06$; +20%), while transpiration was unaffected (Figure 4-4a; Table 4-2). Salinity stress had no effect on stomatal conductance or transpiration (Figure 4-4b and c; Table 4-2).

4.3.2.1.3 UV-B

Ultraviolet-B radiation had no effect on the leaf gas exchange parameters measured (Figure 4-4; Table 4-2).
Figure 4-4. Photosynthesis (a) conductance (b) transpiration and (c) water use efficiency (WUE) (d) of wheat plants grown in solution culture under different Fe, NaCl and UV-B levels. Values are mean ± standard error (n = 6) and WUE data were square root transformed for REML and the graph shows back transformed means and error bars.
Table 4-2. Summary of significance levels from REML analyses describing the effects of Fe deficiency, NaCl and UV-B radiation on leaf gas exchange parameters of wheat plants. Analysis of results water use efficiency (WUE) was based on square root-transformed data.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Photosynthesis (µmol CO₂ m⁻² s⁻¹)</th>
<th>Conductance (mol H₂O m⁻² s⁻¹)</th>
<th>Transpiration (mmol H₂O m⁻² s⁻¹)</th>
<th>WUE (mol CO₂ mol⁻¹H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>***</td>
<td>***</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>NaCl</td>
<td>+</td>
<td>NS</td>
<td>NS</td>
<td>**</td>
</tr>
<tr>
<td>UV-B</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Fe x NaCl</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>*</td>
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<tr>
<td>Fe x UV-B</td>
<td>NS</td>
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<td>NS</td>
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<tr>
<td>NaCl x UV-B</td>
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<tr>
<td>Fe x NaCl x UV-B</td>
<td>NS</td>
<td>NS</td>
<td>+</td>
<td>NS</td>
</tr>
</tbody>
</table>

Raw and transformed data are shown in Appendix 4-1. Significance levels are: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; + $P = 0.05$; NS, $P \geq 0.10$.

4.3.2.2 Interaction effects

4.3.2.2.1 Fe x salt

When no NaCl stress was applied, Fe deficiency decreased WUE by 52% (Figure 4-4d; Table 4-2). Under NaCl stress conditions, Fe deficiency decreased the WUE by 28% (Figure 4-4d; Table 4-2). Under Fe-sufficient conditions, NaCl application did not affect the WUE (Figure 4-4d; Table 4-2). When Fe deficiency conditions were present, the addition of NaCl stress increased WUE by 57% (Figure 4-4d; Table 4-2). There was no interaction between Fe deficiency and NaCl stress on photosynthesis, stomatal conductance or transpiration (Figure 4-4a, b and c; Table 4-2).
4.3.2.2 Fe x UV-B

Under low UV-B radiation levels, Fe deficiency did not affect stomatal conductance (Figure 4-4b; Table 4-2). When high UV-B radiation levels were applied, Fe deficiency decreased stomatal conductance by 42% (Figure 4-4b; Table 4-2). When Fe-sufficient conditions were applied, high UV-B radiation increased stomatal conductance by 52% (Figure 4-4b; Table 4-2). However, under Fe deficiency conditions high UV-B radiation did not affect stomatal conductance (Figure 4-4b; Table 4-2). Additionally, there was no interaction effect from Fe deficiency and high UV-B radiation treatments for photosynthesis, transpiration or WUE (Figure 4-4a, c and d; Table 4-2).

4.3.2.2.3 Other stress interactions

No effect was found for the other two-way (NaCl x UV-B) or three-way (Fe x NaCl x UV-B) stress interactions for leaf gas exchange parameters (Figure 4-4a, b and d; Table 4-2), although transpiration showed a marginally significant effect for the three-way interaction ($P = 0.05$; Figure 4-4c; Table 4-2). The only significant difference in that three-way interaction comparison was that plants exposed to all three stress factors were reduced in their transpiration rates, compared to plants exposed to UV-B and NaCl stress but growing under Fe-sufficiency.

4.4 Discussion

This is the first study examining the three-way interaction of Fe, NaCl and UV-B radiation on a variety of morphological and physiological characteristics of plants. As already noted, little is known specifically about the interaction effects between NaCl stress and UV-B radiation. However, Munns & Tester (2008) have recently suggested that plants grown under NaCl stress would develop similar features and mechanisms to drought stress to enable them to tolerate the low soil water potential of salinity. Additionally, some reports have examined the interaction between drought and UV-B radiation stress on various morphological and physiological characteristic of plants.
Chapter 4

(Alexieva et al., 2001; Balakumar et al., 1993; Cechin et al., 2008; Sullivan & Teramura, 1990), showing features of cross-tolerance between stress factors.

4.4.1 Plant growth and morphology

4.4.1.1 Visual

Chlorosis symptoms on young leaves were observed under Fe deficiency (Figure 4-1; Figure 4-2c and d), similar to those reported by Mengel (1994). It is likely that the interveinal chlorosis was observed on the youngest leaves of Fe-deficient plants happened because the movement of Fe in the phloem is slow and so it is not easily remobilized from the older leaves (Mengel, 1994). Only older leaves showed senescence under NaCl stress (Figure 4-1; Figure 4-2) as re-translocated Na⁺ and Cl⁻ moved to older leaves (Colmer et al., 1995).

It has been reported that increases in exposure to UV-B radiation can have a negative effect on plant growth and other morphological characteristics (Ballare et al., 1999; Searles et al., 1995; Xiong & Day, 2001). However, in this experiment, leaf morphology and gas exchange were not significantly affected by UV-B radiation (Figure 4-1, Table 4-1 and Table 4-2). This is in accordance with a general view that monocots are frequently resistant to UV-B radiation (Cybulski & Peterjohn, 1999; He et al., 1993; Musil & Wand, 1999; Van & Garrard, 1976). In comparison, a study using high solar UV-B irradiation levels at a upper mountain site in the North Chile with German and Chilean wheat showed a decrease in biomass and leaf area but no change in shoot length (Häder, 1996).

Further studies should include the new wheat variety used in this study to compare its UV-B sensitivity with other varieties of wheat.

When plants were subjected to two stresses simultaneously (e.g. Fe x NaCl) the effects of these stresses on plant growth or morphology did not appear to be cumulative (Figure 4-1). Instead, one stress appeared to reduce the effect of the other. Similar results were described by Yousfi et al. (2007) who investigated the combined effects of Fe deficiency and NaCl stress. Yousfi et al (2007) found that the interaction of the two stress factors
alleviated the effect of one stress alone. Such amelioration effects may indicate the conferring of cross-tolerance by one stress factor for the plant response to another stress factor.

### 4.4.1.2 Main effects

#### 4.4.1.2.1 Iron

The reduction of wheat shoot and root growth by Fe deficiency in the research presented here (Figure 4-3a and b; Table 4-1) has also been reported by others (Yousfi et al., 2007; Zhang et al., 1991a, 1991b). The greater reduction in shoot DM compared with root DM in Fe-deficient plants resulted in an increase in the root:shoot in these plants in comparison with Fe-sufficient plants (Figure 4-3c and d; Table 4-1). This occurred under Fe deficiency conditions where roots acquire as much Fe as possible, thus more DM production is allocated to root growth. A similar explanation was suggested by Zhang et al. (1991b). In the current study, leaf area declined considerably under Fe deficiency (Figure 4-3e; Table 4-1) (Bertamini & Nedunchezian, 2005; Dixit & Srivastava, 2000). A large reduction of leaf area may be associated with decreased shoot DM, because leaf area and shoot DM are usually directly proportional to each other (Hofmann et al., 2003). This study showed no effect of Fe-deficient on RWC (Figure 4-3d and e; Table 4-1). This suggests that under Fe-deficient conditions the plants were not under water stress but rather were under growth stress.

#### 4.4.1.2.2 Salt

Yeo et al. (1985) suggested that RWC is a highly sensitive parameter in plants exposed to salinity stress. The fact that the NaCl treatment reduced RWC only slightly (Figure 4-3d; Table 4-1) could suggest that the wheat cultivar tested was under only moderate water stress under the 75mM NaCl treatments. Shoot DM was more sensitive to salinity than root DM, resulting in increased root:shoot (Figure 4-3a, b and c; Table 4-1). This result has also been reported by Bernstain et al. (2001) and Shalhevet et al. (1995). Leaf area was very sensitive to high NaCl levels in the culture solution (Figure 4-3e; Table 4-1), and these results were similar to those reported by Távora et al. (2001) in young guava
plants. This may indicate that as the RWC decreased, cells shrank and cell walls relaxed (Chaves et al., 2003a), causing reduced cell expansion and plant growth. Taken together, these results support the view that the salinity treatment induced physiological drought in the present study.

4.4.1.2.3 UV-B

In this study, plant growth and morphological characteristics were not changed by UV-B radiation (Figure 4-3a and e; Table 4-1). This is similar to the results reported by Zancan et al. (2006) in maize plants. It is commonly stated that plant responses to UV-B radiation can be variable, and that reduction in plant growth only occurs at extremely high levels of UV-B radiation, often paired with low PAR levels (Searles et al., 2001). Given that no plant growth reduction was observed, it suggests that the UV-B treatment used here was not excessive. Fiscus & Booker (1995) suggested that this variability can be observed both within a species and across a range of unrelated species, depending on both UV-B sensitivity and the method of UV-B radiation exposure. The current study showed no effect of UV-B irradiation on RWC (Figure 4-3d; Table 4-1); this was similar to other results in wheat, as shown by Alexieva et al. (2001).

Sunlight does not penetrate the soil surface, yet manipulation of UV-B radiation has been reported to have several consequences to belowground ecosystems (e.g. microbial communities) (Zaller et al., 2002). However, there is little information about the effects of UV-B on roots in wheat or related species. This study showed slightly decreased root DM and root:shoot under UV-B (Figure 4-3b and c; Table 4-1). This suggests that UV-B radiation might indirectly reduce root growth but not affect shoot growth, thus inducing decreased root:shoot in this wheat cultivar. It is possible that the acclimatization of the plants to the aboveground UV-B stress came at a cost for root growth, perhaps due to a lesser allocation of resources to the root zone.
4.4.1.3 Interaction effects

4.4.1.3.1 Fe x Salt

In this study, wheat plants exposed to salinity stress were less sensitive to Fe stress than plants not exposed to salinity, as shown by the plant growth and morphological measurements (Figure 4-3a, b, and e; Table 4-1). This was also reported by Yousfi et al. (2007) in barley. Hofmann et al. (2003) found that co-occurring stress factors can provide cross-tolerance, where one stress protects plants against another stress, thus mitigating the total effects of stress.

4.4.2 Gas exchange in leaves

4.4.2.1 Main effects

4.4.2.1.1 Iron

Iron deficiency decreased all gas exchange parameters, such as photosynthetic rates, stomatal conductance, transpiration rates and WUE (Figure 4-4; Table 4-2). This result was similar to that described by Spiller & Terry (1980), who stated that photosynthesis processes were Fe dependent; for example, the cytochrome b$_{6}$f complex and of the Fe-S protein of the photosynthetic electron transport chain. Other research also suggests that Fe deficiency may be associated with decreased stomatal conductance (Molassiotis et al., 2006), reducing the transpiration rates and intercellular CO$_{2}$ concentration. This may lead to decreased Calvin Cycle activity, photosynthesis rates and WUE (Molassiotis et al., 2006) as also observed in this study (Figure 4-4; Table 4-2). Furthermore, the reduction in photosynthetic rates under Fe deficiency will be affected by the pronounced level of chlorosis observed in that treatment (Figure 4-1).
4.4.2.1.2 Salt

There was no change in stomatal conductance and transpiration rates under NaCl stress (Figure 4-4b and c; Table 4-2). Whereas WUE increased under NaCl stress (Figure 4-4d; Table 4-2). In general levels of NaCl stress of 100 mM NaCl or more are likely to decrease transpiration and photosynthesis rates, and increase WUE, as strongly decreased RWC under high salinity could result in stomatal closure to improve the water status of the plant (Hasegawa et al., 2000; Nunes et al., 2008; Stępień & Kłobus, 2006). Similar to the findings for RWC (-5%) under NaCl treatment (Figure 4-3d; Table 4-1), the gas exchange results from this study suggest that the NaCl stress applied here was moderate.

4.4.2.1.3 UV-B

Ultraviolet-B radiation did not affect any leaf gas exchange parameters measured. This is consistent with previous studies that have shown that photosynthesis parameters are only affected when plants are exposed to excessive UV-B radiation levels (Allen et al., 1998; Bassman et al., 2002).

4.4.2.2 Interaction effects

4.4.2.2.1 Fe x salt

To the best of the author’s knowledge, this is the first investigation of WUE responses in plants subjected to combined NaCl and Fe stress. The decrease in WUE under Fe deficiency can be explained by a stronger effect of that stress on photosynthesis, compared to transpiration (Figure 4-4a, c and d; Table 4-2). Under optimal Fe levels, NaCl stress did not affect WUE, again highlighting the moderate nature of the NaCl stress applied (Figure 4-4d; Table 4-2). However, WUE increased under Fe deficiency conditions and NaCl stress compared with no NaCl stress (Figure 4-4d; Table 4-2), suggesting that NaCl stress can alleviate Fe deficiency-driven decreases in WUE.
4.4.2.2  Fe x UV-B

The findings of this study revealed that the Fe deficiency-induced decreases in stomatal conductance only occurred when UV-B radiation was present (Figure 4-4b; Table 4-2), showing that the presence of that additional stress needs to be considered in Fe deficiency studies. The UV-B-induced increase in stomatal conductance under Fe sufficient conditions (but not when Fe was limited) (Figure 4-4a; Table 4-2) is supported by other findings, where UV-B resulted in an opening of the stomata (Jansen & van den Noort, 2000; Musil & Wand, 1993; Teramura et al., 1983).

4.5  Conclusion

The results showed that Fe deficiency and NaCl stress significantly affected plant growth and morphology, while UV-B radiation had some effects but only on belowground parameters. Gas exchange was not affected by UV-B radiation and only slightly by salinity, while most gas exchange parameters were strongly affected by Fe deficiency. While most stress interactions did not alter leaf gas exchange parameters, the findings of this study could suggest that the Fe status of plants may need to be considered when investigating NaCl or UV-B stress effects on gas exchange parameters in plants.
5 Effects of induced stress on biochemical attributes¹

5.1 Introduction

Plants under environmental stress alter their metabolism, for examples by changing their leaf pigment and amino acid content to acclimatize to the stress. For instance, Fe deficiency leads to interveinal chlorosis in young leaves because of a reduction in leaf chlorophyll content (Ksouri et al., 2005). Specific mechanisms of Fe acquisition in higher plants have been grouped into two strategies: Strategy I and II (Römheld & Marschner, 1986). Wheat is a Strategy II plant (as are other Poaceae species) with a distinct diurnal release of nonproteinaceous amino acids such as mugineic acids, called phytosiderophores (PSs). Phytosiderophores are released from the roots to solubilise Fe compounds in the rhizosphere (Zhang et al., 1991b). Recently, both temperature and light have been suggested as being involved in the regulation of diurnally secreted PSs in barley (Reichman & Parker, 2007; Takagi, 1990). However, there has been a lack of research on the effects of multiple environmental stresses on the secretion of PSs by plants.

Salinity stress mainly reduces plant growth due to osmotic and ionic effects (Munns, 2002). Plants respond to NaCl stress by accumulating organic solutes to reduce water potential and therefore stimulate the flow of water into cells and also to protect sensitive compounds and structures in cells (Robinson et al., 1997; Serraj & Sinclair, 2002). A particular candidate in this regard is the amino acid proline which, in its free form, can act as an osmoprotectant (Binzel et al., 1987; Ketchum et al., 1991).

Enhanced levels of UV-B radiation ultimately result in changes that modify the penetration of UV radiation into plants and induce structural and biochemical changes. In general, plants synthesise UV-absorbing compounds (usually phenolic compounds) in epidermal cells as UV-screening pigments (Cen & Bornman, 1993). This response is

¹ This chapter has been published as Wong et al. (2008) in Presentation at the Chemistry and Biosphere Conference and is formatted accordingly.
important for reducing the penetration of UV-B radiation to underlying tissues. However, a proportion of UV-B nevertheless can enter into leaf and this may result in a number of effects, including a reduction in the levels of some amino acids (Moorthy & Kathiresan, 1998).

There have been few research studies on UV-B radiation effects on iron content or iron treatments (De la Rosa et al., 2001; Zancan et al., 2006; Zancan et al., 2008). Recent studies have shown that PS release is mediated by light not temperature (Reichman & Parker, 2007). It is therefore reasonable to test how other spectral wavelengths affect PSs in roots and how this interacts with iron deficiency. There is only limited information on PS release in combination with UV-B and Fe deficiency stress on plants (Zancan et al., 2008). In addition, there has been no research on the interaction between Fe nutrition stress, NaCl stress and UV-B exposure on plants.

The general objective of this chapter was to determine aboveground and belowground aspects of plant biochemical responses under Fe, salinity and UV-B stress. The specific aim of this study is to investigate how three stress factors, Fe, NaCl and UV-B, affect the biochemical attributes of wheat and whether UV-B radiation will directly enhance levels of UV absorbing compounds and indirectly diminish the release of amino acid-derived PSs belowground. An additional aim was to investigate the effects that Fe, NaCl and UV-B radiation have on chlorophyll and proline levels. It was hypothesised that these biochemical attributes will be differentially affected by the three stress factors and their interactions.
5.2 Materials and methods

The wheat plants were grown according to the experimental design, as outlined in Chapter 3. Additional details about the methods of analyses employed are presented in this chapter.

Chlorophyll content of wheat plants

Extraction of chlorophyll from wheat laminae was performed in N,N-dimethylformamide (DMF) (Moran & Porath, 1980). Plant samples were ground, freeze-dried and subsamples weighed (10 mg) into centrifuge tubes, as described in Chapter 3. Samples were extracted with 1.2 ml DMF in darkness at 4°C for 24 h under occasional vortex shaking. This was followed by centrifugation at 13 500 rpm for three minutes. Of the supernatant, 50μl was diluted with 1 ml DMF and placed in a quartz cuvette. Absorbance of the supernatant was read immediately at wavelengths of 664.5 nm and 647 nm and chlorophyll a and b content was calculated using the following equations (Inskeep & Bloom, 1985):

Chlorophyll a (mg g⁻¹ DM) = 12.7 A₆₆₄.₅ – 2.79 A₆₄₇
Chlorophyll b (mg g⁻¹ DM) = 20.7 A₆₄₇ – 4.62 A₆₆₄.₅
Total Chlorophyll (mg g⁻¹ DM) = 17.9 A₆₄₇ + 8.08 A₆₆₄.₅

Where A₆₄₇ refers to the absorbance at 647 nm and A₆₆₄.₅ to the absorbance at 664.5 nm.

UV-absorbing compounds of wheat plants

The concentration of UV-absorbing compounds was determined using established procedures (Mirecki & Teramura, 1984). Freeze-dried samples were ground in liquid nitrogen and weighed (15 mg) into 1.5 ml centrifuge tubes. Extraction in 1.2 ml MeOH: H₂O: HCl (79:20:1) occurred in darkness for 24 h and samples were vortexed at regular intervals. This was followed by centrifugation at 10 000 rpm for 3 min and subsequent spectrophotometric analysis of 50 μl supernatant diluted with 3 ml methanol (MeOH) in a quartz cuvette. The absorbance was read at 300 nm and results were presented as leaf DM mg ml⁻¹.
**Phytosiderophores in roots**

Root exudate phytosiderophores (PSs) samples were collected using established procedures (Reichman & Parker, 2007). The PSs were collected between 2 and 5 h after the light period commenced, because PSs in wheat has been previously determined to be optimum during this period (Ma & Nomoto, 1996). The lid of each container containing the plants was carefully removed from the nutrient solution so as not to damage the plants. The plants were rinsed twice with RO water to clean the roots, then transferred to 750 ml plastic collection containers filled with 250 ml deionised water so the roots were covered by the collection solution. The plants were left in the growth chamber under their radiation treatment regimes for three hours. After the collection period the plants were gently removed from the collection container and returned to the nutrient solution. To prevent microbial degradation of the PSs, 1 ml of thymol (15 mg l⁻¹) was added to the collected exudates upon removal of the plants (Shen et al., 2001). This was followed by vacuum-filtration through a 0.45 µm filter. Samples were stored in a freezer (-20 °C) until further analysis.

Iron-binding ligands in the root exudates previously demonstrated to be quantitatively equivalent to PSs in wheat (Reichman & Parker, 2007) were quantified by the revised Fe-binding assay (Reichman & Parker, 2006). Briefly, 10 ml of sample was mixed with 0.5 ml of 0.6 mM FeCl₃ and shaken for 15 min. Then 1 ml of 1.0 M Na-acetate buffer (pH 7.0) was added and the solution and shaken for ten min. After shaking, the solution was filtered through a 0.2 µM Whatman GF/F filter paper into 0.25 ml of 6 M HCl, before the addition of 0.5 ml of 80 g l⁻¹ hydroxylamine hydrochloride. Solutions were placed in an oven at 50-60°C for 30 min. Upon removal, 0.25 ml of 2.5 g l⁻¹ ferrozine and 1 ml of 2.0 M Na-acetate buffer (pH 4.7) were added and the contents mixed. After 5 min the absorbance was determined at 562 nm using a Philips PU8700 Series UV/V spectrophotometer (Philips Analytical, Cambridge, United Kingdom). Absorbances were converted to concentrations using the Beer-Lambert law (Tam & Zardecki, 1982).
Proline in wheat plants

Free proline was determined in fully open wheat laminae using established methods (Magné & Larher, 1992). Each sample was ground in liquid nitrogen, freeze-dried and then 10 mg was weighed into centrifuge tubes. Protein was precipitated from the samples by the addition of 1.2 ml of 3% (w v⁻¹) sulphosalicylic acid under vortex shaking, followed by centrifugation at 10 000 rpm for 7 min. The supernatant was removed, centrifuged again (10 000 rpm) and 500 µl of the resulting supernatant collected and made up to 1 ml with deionised water. Two ml ninhydrin agent (1% (w v⁻¹) ninhydrin in 60% (v v⁻¹) glacial acetic acid) were added to the supernatant and the sample stored at 4°C overnight. The following day, samples were vortex-shaken and the solution left to react for 1 h at 98°C. The reaction was then slowed by first placing sample tubes in an ambient-temperature water-bath for two min. and then halted by placing the sample tubes in an ice-water bath for another 2 min., followed by addition of 2 ml toluene and vortex shaking for 20 sec. Phases were allowed to separate for at least 5 min and the products extracted into the toluene phase were examined spectrophotometrically in a 1 ml quartz cuvette at 520 nm. Free proline content in the wheat samples was calculated from a standard curve of known proline concentrations (0 to 25 µg ml⁻¹).
5.3 Results

5.3.1 Leaf pigment content

5.3.1.1 Main effects

5.3.1.1.1 Iron
Iron-deficiency induced reductions in all chlorophyll (Chl) fractions: Chl total (-61%), Chl a (-65%) and Chl b (-31%) compared to the Fe-sufficient treatment (Figure 5-1a, b and c; Table 5-1). In contrast, the Chl a:b decreased by 25% and UV-absorbing compounds increased by 5% in response to Fe deficiency (Figure 5-1d and e; Table 5-1).

5.3.1.1.2 Salt
Salinity stress reduced all the chlorophyll fractions, such as Chl total (-13%), Chl a (-14%) and Chl b (-5%) and Chl a:b ratio (-6%) compared to the no NaCl treatment (Figure 5-1a, b, c and d; Table 5-1). However, NaCl stress had no effect on UV-absorbing compounds (Figure 5-1e; Table 5-1).

5.3.1.1.3 UV-B
Exposure of wheat plants to UV-B radiation had no significant effect on the contents of the chlorophyll fraction: Chl total, Chl a, Chl b, or the Chl a:b ratio (Figure 5-1a, b, c and d; Table 5-1). However, high UV-B radiation increased UV-absorbing compounds by 38% in comparison to the low UV-B control treatment (Figure 5-1 e; Table 5-1).
Figure 5-1. Chlorophyll (Chl) total (a), Chl a (b), Chl b (c), Chl a:b (d) and UV-absorbing compounds (e) levels of wheat plants grown in culture solution under different Fe, NaCl and UV-B radiation levels. Values are mean ± standard error (n = 6), the Chl b data were square root transformed before REML analysis and the graph show back-transformed means and error bars.
Table 5-1. Summary of significance levels from REML analysis describing the effects of Fe deficiency, NaCl stress and UV-B radiation on plant leaf pigment content of wheat plants. Chl, chlorophyll; Chl b analysis was based on square root transformed data.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Chl total (mg g⁻¹ DM)</th>
<th>Chl a (mg g⁻¹ DM)</th>
<th>Chl b (mg g⁻¹ DM)</th>
<th>Chl a:b</th>
<th>UV-absorbing compounds (mgDMml⁻¹)</th>
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<tr>
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<td></td>
<td>NS</td>
</tr>
<tr>
<td>Fe x NaCl x UV-B NS NS NS NS NS</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Raw and transformed data are shown in Appendix 5-1. Significance levels are: *** \( P < 0.001 \); ** \( P < 0.01 \); * \( P < 0.05 \); + \( P < 0.1 \); NS, \( P \geq 0.10 \).

5.3.1.2 Interaction effects

5.3.1.2.1 Fe x UV-B

Under low levels of UV-B irradiation, Fe deficiency had no significant effect on levels of UV-absorbing compounds in wheat leaves (Figure 5-1e; Table 5-1). When high UV-B levels were applied, Fe deficiency increased UV-absorbing compounds (+10%) in wheat leaves (Figure 5-1e; Table 5-1). Under Fe-sufficient conditions, UV-B radiation increased UV-absorbing compounds by 31% in wheat leaves (Figure 5-1e; Table 5-1). Under Fe deficiency conditions, additional UV-B radiation increased levels of UV-absorbing compounds by 45% in wheat leaves (Figure 5-1e; Table 5-1). There was no significant interaction between Fe deficiency and UV-B radiation on plant chlorophyll fractions (Figure 5-1a, b, c and d; Table 5-1).
5.3.1.2.2 Other stress interactions

No significant effect was found for the other two-way (Fe x NaCl and NaCl x UV-B stress) or three-way (Fe x NaCl x UV-B stress) interactions for leaf pigment content (Figure 5-1; Table 5-1), although the Chl a:b ratio did show a marginally significant \((P = 0.07)\) effect under the combination of Fe deficiency and NaCl stress in wheat leaves (Figure 5-1d; Table 5-1). This interaction showed Fe deficiency-generated decreases of around 25% for the Chl a:b ratio under both salinity treatments, while salt-induced decreases in the Chl a:b ratio were only observed under Fe deficiency (-12%), but not under Fe sufficiency.

5.3.2 Amino acids

5.3.2.1 Main effects

5.3.2.1.1 Iron

Iron-deficiency increased PS release from roots 2.4 fold but had no significant effect on proline accumulation in wheat leaves (Figure 5-2a and b; Table 5-2).

5.3.2.1.2 Salt

Salinity stress generated a large, 16.3 fold increase in proline accumulation compared to the no-NaCl treatment (Figure 5-2b; Table 5-2). However, NaCl stress had no effect on PS release from roots (Figure 5-2a; Table 5-2).

5.3.2.1.3 UV-B

Exposure of wheat plants to high UV-B radiation showed a marginally significant \((P = 0.05)\) decrease in the secretion of PSs by 47%, but no significant effect on proline accumulation, when compared to the low UV-B control treatment (Figure 5-2a and b; Table 5-2).
Chapter 5

Figure 5-2. Phytosiderophores released from roots (a) and proline levels in shoots (b) of wheat plants grown in culture solution under different Fe, NaCl and UV-B radiation levels. Values are mean ± standard error (n = 6).
Table 5-2. Summary of significance levels from REML analyses describing the interaction of Fe deficiency, NaCl stress and UV-B radiation on amino acid content of wheat plants.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Phytosiderophores (µmol g⁻¹ root DW)</th>
<th>Proline (µg mg⁻¹ DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>***</td>
<td>NS</td>
</tr>
<tr>
<td>NaCl</td>
<td>NS</td>
<td>***</td>
</tr>
<tr>
<td>UV-B</td>
<td>+</td>
<td>NS</td>
</tr>
<tr>
<td>Fe x NaCl</td>
<td>+</td>
<td>NS</td>
</tr>
<tr>
<td>Fe x UV-B</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>NaCl x UV-B</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Fe x NaCl x UV-B</td>
<td>+</td>
<td>NS</td>
</tr>
</tbody>
</table>

Raw data are shown in Appendix 5-1. Significance levels are: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; + $P < 0.1$; NS, $P \geq 0.10$.

5.3.2.2 Interaction effects

5.3.2.2.1 Fe x Salt

There was a marginally significant interaction between Fe deficiency and NaCl stress for PS secretion (Figure 5-2a; Table 5-2). When no NaCl stress was imposed, the additional application of Fe deficiency increased PS release 3.5 fold compared to the Fe-sufficiency treatment (Figure 5-2a; Table 5-2). Under NaCl stress conditions, Fe deficiency only increased PS release from roots by 76% compared to the Fe sufficiency treatment (Figure 5-2a; Table 5-2). However, there was no significant effect of NaCl stress on PS release under either of the two Fe treatments (Figure 5-2a; Table 5-2). There was also no effect from the interaction between Fe deficiency and NaCl stress for proline accumulation (Figure 5-2b; Table 5-2).
5.3.2.2 Other stress interactions

No significant effect was found for the other two-way (Fe x UV-B and NaCl x UV-B stress) or three-way (Fe x NaCl x UV-B stress) interactions for proline accumulation (Figure 5-2a and b; Table 5-2). Phytosiderophores release from the roots showed a marginally significant ($P = 0.09$) three-way interaction effect (Figure 5-2a; Table 5-2). Under conditions of no NaCl, Fe deficiency increased PS release 5.3-fold under high UV-B radiation and 3-fold under low UV-B radiation, respectively (Figure 5-2a, Table 5-2). However, under conditions of imposed NaCl stress, Fe deficiency increased PS release 2.6-fold under high UV-B radiation but there was no change in PSs release under low UV-B radiation conditions (Figure 5-2a, Table 5-2). Finally, under Fe-sufficient conditions, the imposition of NaCl stress resulted in no change in PS release under both high UV-B and low UV-B radiation treatments (Figure 5-2a, Table 5-2).
5.4 Discussion

5.4.1 Leaf pigment content

5.4.1.1 Main effects

5.4.1.1.1 Iron

The reduction in wheat chlorophyll concentration in response to Fe deficiency (Figure 5-1; Table 5-1) is a well-known response (Rabhi et al., 2007; Zhang et al., 1991b). In this current study, the chlorophyll concentration of young leaves was affected especially by Fe deficiency (figure 4-1; Figure 4-2). This could explain the appearance of leaf chlorosis in wheat exposed to Fe-deficient conditions, a characteristic of Fe-deficient plants (Ksouri et al., 2005). Iron is necessary for chlorophyll biosynthesis as well as thylakoid and granum formation (Spiller & Terry, 1980). Additionally, results showed increased UV-absorbing compounds under Fe deficiency (Figure 5-1e; Table 5-1). This is similar to the results reported for studies on barley plants (Zancan et al., 2008). However, it could also be suggested that the increase in UV-absorbing compounds represented a general nutrient stress response, rather than being specific to Fe deficiency, as UV-absorbing compounds have also been shown to increase in response to UV-B under other nutrient stress conditions, e.g. low nitrogen levels in cucumber leaves (Hunt & McNeil, 1998).

5.4.1.1.2 Salt

Salinity stress decreased chlorophyll concentration in the wheat leaves (Figure 5-1; Table 5-1). This is similar to the results reported in studies of cucumber plants (Stepień & Klobus, 2006; Sultana et al., 1999), and might be attributed to both an increased degradation and inhibited synthesis of chlorophyll due to NaCl toxicity (Sultana et al., 1999).
5.4.1.1.3 UV-B

In the current study, no effects of UV-B radiation on chlorophyll concentration were found (Figure 5-1; Table 5-1). These results are similar to the results reported by Cechin et al. (2008) and Hofmann et al. (2003) in sunflower and white clover, respectively. These results, however, contradict findings by Alexieva et al. (2001) who showed a significant decrease in chlorophyll concentration for wheat and pea plants grown under elevated UV-B. Pradhan et al. (2006) suggested that Alexieva et al. (2001) used very low PAR levels and high UV-B levels; conditions that might increase the degree of UV-B-induced loss of chlorophyll due to cellular damage.

In the current study, UV-B radiation increased the concentration of UV-absorbing compounds in wheat shoots compared to the low UV-B control treatment (Figure 5-1e; Table 5-1). This is similar to other observations, for example in Hofmann et al. (2003) in white clover plants. Ultraviolet-absorbing compound accumulation is seen to provide effective UV-B protection to plants (Hofmann et al., 2003; Jordan, 2002).

5.4.1.2 Interaction effects

5.4.1.2.1 Fe x UV-B

Among the stress factors studied here, Fe deficiency affected chlorophyll concentration most strongly (Figure 5-1a, b, c and d; Table 5-1), while UV-B radiation had the biggest impact on the accumulation of UV-absorbing compounds (Figure 5-1e; Table 5-1). In addition, accumulation of UV-absorbing compounds was synergistically enhanced under the combination of elevated UV-B radiation and Fe deficiency stress. A positive stress interaction for combined UV-B and Fe stress has also been reported by Zancan et al. (2008) for the accumulation of UV-absorbing compounds. These synergistic responses may be due to the elicitation of common stress regulatory factors (Logemann & Hahlbrock, 2002).
5.4.2 Amino acids

5.4.2.1 Main effects

5.4.2.1.1 Iron

Increased levels of PSs released from wheat roots in response to Fe deficiency, as found in the current study (Figure 5-2a; Table 5-2), have frequently been reported; for example, Reichman & Parker (2007) and Römheld & Marschner (1990). This is a feature of roots in strategy II plant species which typically respond to Fe deficiency by the synthesis and release of PSs to solubilise Fe (Römheld, 1987). This study showed no effect of Fe deficiency on proline concentrations (Figure 5-2b; Table 5-2). This is not surprising as proline accumulation is mainly a function of osmotic stress factors (Binzel et al., 1987; Ketchum et al., 1991) not Fe deficiency.

5.4.2.1.2 Salt

Of the two amino acids examined, proline was the only stress marker measured that increased under NaCl stress (Figure 5-2b; Table 5-2). This is similar to results reported by Alexieva et al. (2001) in pea and wheat plants, by Balakumar et al. (1993) in cowpea and by Nayyar & Walia (2003) in wheat. Under NaCl stress, plants induce proline accumulation in the cytosol to buffer cellular redox potential (Ashraf & Foolad, 2007). This suggests that under NaCl stress conditions, high accumulation of proline (16.3 fold increases in the current study) can contribute to osmotic adjustment and water status in wheat plants (Nayyar & Walia, 2003). This can help maintain normal plant function under stress and is supported by the observation that photosynthesis rates were not decreased under salt stress (Figure 4-4a; Table 4-2) and the reduction in RWC was very low (Figure 4-3d; Table 4-1). Release of PSs was not affected by the NaCl stress applied here (Figure 5-2a; Table 5-2), and this is in agreement with other studies that showing that the release of PSs is a function of Fe deficiency, rather than NaCl stress (Yousfi et al., 2007).
5.4.2.1.3 UV-B

In this study, UV-B radiation resulted in a marginally significant decrease in PS release (Figure 5-2a; Table 5-2). As far as the author is aware, no previous research has investigated UV-B radiation affects on the secretion of PSs in wheat. Other research studies have focused on the effects of photosynthetically active radiation (PAR) on PS release by roots. For example, Reichman & Parker (2007) demonstrated that the release of PSs in wheat is triggered by changes in PAR. The findings reported here are consistent with other research that found UV-B radiation generated decreases of amino acid levels in plants (Moorthy & Kathiresan, 1998), possibly due to UV-B-induced changes in gene expression. The results reported here therefore suggest that in PAR addition to UV-B radiation is important for PS release in wheat.

This study showed no effect of UV-B radiation on the accumulation of proline in wheat plants (Figure 5-2b; Table 5-2). These results differ from those of Hofmann et al. (2003) in white clover and Shetty et al. (2002) in other legumes who showed UV-B-induced enhancement of proline accumulation. Proline can be seen in the first instance as an osmoprotectant while its role in UV protection is less clear (Hofmann et al., 2003). Monocotyledonous plants are frequently seen to be less responsive to UV-B than dicots, due to a number of morphological and physiological features, including leaf architecture (Rozema et al., 1997) and this may help explain the lack of proline response in wheat grown under increased UV-B.
5.4.2.2 Interaction effects

5.4.2.2.1 Fe x Salt

Iron deficiency strongly increased PS release when NaCl stress was not present, while this increase was less pronounced when NaCl stress was applied in conjunction with Fe deficiency (Figure 5-2 a; Table 5-2). This is similar to the results reported by Yousfi et al. (2007) after exposure of barley plants to three weeks of Fe deficiency and NaCl stress. Yousfi et al. (2007) showed that NaCl stress may inhibit PS release induced by Fe deficiency in barley at week two because NaCl stress may increase root senescence and the disappearance of active root zones which indirectly induce PSs synthesis and release in barley roots. They suggested that while there was no growth reduction in barley roots exposed to 100 mM NaCl, there was a decrease of PSs release, indicating a NaCl inhibition of PSs release. It must be pointed out that these authors used higher salinity levels compared to the current study and it is possible that the conditions used by Yousfi et al. (2007) may have increased the degree of water stress-induced loss of root growth.

5.4.2.2.2 Fe x Salt x UV-B

In this study, the three-way interaction between Fe, NaCl and UV-B radiation indicated a marginally significant effect on PSs release and no effect on the accumulation of proline in wheat plants (Figure 5-2a and b; Table 5-2). This experiment showed that under Fe deficiency conditions alone, (-Fe x –NaCl x –UV-B), PS release was more pronounced than for plants grown under the interaction of Fe deficiency with NaCl and high UV-B radiation (-Fe x +NaCl x +UV-B) treatments (Figure 5-2a and b; Table 5-2). This suggests that the combined NaCl and UV-B radiation treatments can reduce PSs release. As discussed above (section 5.4.2.2.1), NaCl treatments tend to increase root senescence and reduce active root zones (Yousfi et al., 2007); however, as discussed above (section 5.4.2.1.3) UV-B radiation tends to decrease amino acids in plants (Moorthy & Kathiresan, 1998), both of which could help explain the results found here.
5.5 Conclusions

The results from this study showed that important biochemical parameters can be affected by the three stress factors and by their combined application. Chlorophyll content was reduced by Fe deficiency and salinity, but not by UV-B radiation or by two-way or three-way interactions of these stress factors in wheat plants. In contrast, UV-absorbing compounds in wheat were enhanced by UV-B radiation and Fe deficiency and further by the two-way interaction between Fe deficiency and UV-B radiation. Phytosiderophore secretion increased under Fe deficiency and decreased under UV-B radiation, but there was no effect of NaCl stress on PS release. Iron-deficiency induced Fe release, particularly when no NaCl stress was present. Additionally, Fe deficiency conditions, applied together with NaCl and high UV-B radiation (-Fe x +NaCl x +UV-B) tended to reduce PSs release compared with plants grown under no NaCl and low UV-B radiation (-Fe x –NaCl x –UV-B). This study also showed that free proline accumulation was decreased only by salinity stress.
Chapter 6

6 Effects of induced stress on nutritive value

6.1 Introduction

Wheat (Triticum aestivum L.) is an important crop for grain production and is sometimes even used as a forage crop (Cherney & Marten, 1982). Iron deficiency in wheat reduces chlorophyll levels, with a concomitant reduction in photosynthesis (Figure 4-4 and Figure 5-1; Table 4-2 and Table 5-2 from previous chapter), which could result in reduced sugar levels. However, there is little information about the effect of Fe deficiency on feed value and studies are required in this area. Under saline conditions, organic solute accumulation helps maintain homeostasis and stabilizes macromolecules and cell components such as proteins, protein complexes and cell membranes (Bohnert & Shen, 1999). There is lack of studies investigating how salinity stress affects nutrient levels and feed quality parameters such as crude protein, water-soluble carbohydrate (WSC) and acid detergent fibre (ADF) content. In view of the impact of enhanced UV-B radiation on plant physiology, morphology, growth, nutrition, biomass and leaf quality have been investigated extensively (Ballare et al., 1999; Hofmann, 2000; Li et al., 1998; Zu et al., 2004). Little is known, however, about the effects of enhanced UV-B radiation on feed value. Additionally, there has been no research that focused on two- and three-way interactions of these stress factors on nutritive value of plants.

The aim of this study was to investigate how three stress factors, Fe deficiency, NaCl and UV-B radiation, would affect the nutritive value of wheat. The objective of this chapter was to determine aboveground and belowground aspects of plant nutritive value in response to these stress factors. It was hypothesised that these attributes will be differentially affected by the three stress factors and their interactions, resulting in cross-tolerance of plants exposed to the combination of stress factors, when compared with plants exposed to one stress factor alone.
6.2 Materials and methods

The plants were grown using the experimental design, as outlined in chapter 3, with additional details of the methods of analysis presented in this chapter.

Feed value
A FOSS near-infrared (NIR) systems model 5000 monochromator (FOSS NIR, Silver Spring, Maryland, USA) was used to obtain the near-infrared reflectance (NIR) spectra of each dried, ground sample using the ISIscan software (Kong et al., 2005). Each ground sample was placed in a ring cup (35 mm inner diameter, 8 mm depth) covered fully by the sample, then scanned using the wavelength range of 1100-2498 nm. Samples were examined by NIR for crude protein, WSC and ADF (Kong et al., 2005). The percentage of nitrogen was calculated as the crude protein in the sample divided by 6.25 (Horowitz, 1975).

Nutrient content
Nutrient concentrations in wheat shoots and roots were determined using established methods (Gray et al., 1999). Each freeze-dried sample was ground and then 500mg sample material was weighed into separate digestion tubes. A blank and a reference sample of tomato leaves (1573a of National Institute of Standards and Technology (NIST)) were included in each run. After 10 ml of 70% HNO3(aq) was added to each digestion tube, the tubes were placed on a hot plate in a digestion chamber and left overnight. After cooling, the solutions were filtered through Whatman No.52 filter paper (Whatman International Ltd, Maidstone, England) and quantitatively transferred to 25 ml volumetric flasks, then made up to the mark with double-deionised water. The digestions were then stored at 4°C until further analysis. Aliquots of the filtered digestion solutions were analysed by a Varian 720-ES Inductively Coupled Plasma Optical Emission Spectrometer (Varian, ICP-OES, Melbourne, Australia) for the determination of Fe concentrations in the shoot and root tissues.
6.3 Results

6.3.1 Main effects

6.3.1.1 Iron
Iron deficiency decreased crude protein% (-13%), WSC% (-37%), Fe concentration in shoots (-51%), Fe concentration in roots (-45%) and increased ADF% (+5%) compared to the Fe-sufficient treatments (Figure 6-1; Table 6-1).

6.3.1.2 Salt
Salinity stress reduced levels of crude protein% (-5%), WSC% (-48%), ADF% (-2%), Fe concentration in roots (-6%) and increased Fe concentration in shoots (+21%) compared to the no-NaCl treatments (Figure 6-1; Table 6-1).

6.3.1.3 UV-B
None of the feed value parameters analysed was altered by exposure of the wheat plants to UV-B radiation (Figure 6-1a, b and c; Table 6-1). In contrast, Fe concentration in shoots increased by 12% and Fe concentration in roots was marginally reduced ($P = 0.08$) by 7% under the UV-B radiation treatments (Figure 6-1 d and e; Table 6-1).
Figure 6-1. Crude protein (a), water-soluble carbohydrates (WSC) (b), acid detergent fibre (ADF), Fe concentration in shoots (d) and Fe concentration in roots (e) of wheat plants grown in culture solution under different Fe, NaCl and UV-B radiation treatments. Values are mean ± standard error (n = 6).
Table 6-1. Summary of significance levels from REML analyses describing the interaction of Fe deficiency, NaCl stress and UV-B radiation on selected nutritive value in wheat plants. WSC, water-soluble carbohydrates and ADF, acid detergent fibre.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Crude protein (%)</th>
<th>WSC (%)</th>
<th>ADF (%)</th>
<th>Fe in shoot (µg g⁻¹ DM)</th>
<th>Fe in root (µg g⁻¹ DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>***</td>
<td>***</td>
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<td>Fe x NaCl x UV-B</td>
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<td>NS</td>
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<td>**</td>
</tr>
</tbody>
</table>

Raw data are shown in Appendix 6-1. Significance levels are: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; + $P < 0.10$; NS, $P \geq 0.10$.

### 6.3.2 Interaction effects

#### 6.3.2.1 Fe x NaCl

When no NaCl stress conditions were present, Fe deficiency did not affect levels of crude protein% (Figure 6-1a; Table 6-1) but increased ADF% by 3% (Figure 6-1c; Table 6-1), as well as decreasing Fe concentration in shoots (-42%) and roots (-37%) (Figure 6-1d and e; Table 6-1). When NaCl stress conditions were applied, Fe deficiency reduced crude protein% (-24%), Fe concentration in shoots (-58%) and roots (-54%) (Figure 6-1a, d and e; Table 6-1) but increased ADF% by 7% (Figure 6-1c; Table 6-1). Under Fe sufficient conditions, the NaCl stress applied increased crude protein% and Fe concentration in shoots by 7% and 34%, respectively (Figure 6-1a and d; Table 6-1) and decreased ADF% by 4% (Figure 6-1c; Table 6-1), but there was no change in Fe concentration in roots (Figure 6-1e; Table 6-1). However, under Fe deficiency conditions the additional NaCl stress decreased crude protein% and Fe concentration in the roots by 18% and 23%, respectively (Figure 6-1a and e; Table 6-1), but had no effect on ADF% and Fe concentration in shoots (Figure 6-1c and d; Table 6-1). Additionally, there was no interaction effect of the Fe x NaCl treatment combination on WSC% (Figure 6-1b; Table 6-1).
6.3.2.2 Fe x UV-B

Under low UV-B conditions, Fe deficiency decreased both Fe concentration in shoots and roots by 46% and 40%, respectively (Figure 6-1d and e; Table 6-1). When high UV-B radiation conditions were applied, Fe deficiency decreased Fe concentration in shoots (-55%) and roots (-50%) (Figure 6-1d and e; Table 6-1). Under Fe sufficient conditions, the additional high UV-B radiation increased Fe concentration in shoots by 19% (Figure 6-1d; Table 6-1), but there was no change in Fe concentration in roots (Figure 6-1e; Table 6-1). Under Fe deficiency conditions high UV-B had no effect on Fe concentration in shoots (Figure 6-1d; Table 6-1), but decreased Fe concentration in roots by 19% (Figure 6-1e; Table 6-1). Additionally, there was no interaction effect of the Fe x UV-B treatment combination for crude protein%, WSC% or ADF% (Figure 6-1a, b and c; Table 6-1).

6.3.2.3 Other stress interactions

No effects were found for other two-way (NaCl x UV-B stress) or three-way (Fe x NaCl x UV-B stress) interactions for selected nutritive values (Figure 6-1; Table 6-1), except for Fe concentration in roots, which showed a significant three-way interaction effect (Figure 6-1e; Table 6-1):

6.3.2.3.1 Fe effect, dependent on salt and UV-B treatments

Under no NaCl conditions, Fe deficiency showed decreased Fe concentration in roots by 48% at high UV-B radiation and 25% at low UV-B radiation (Figure 6-1e; Table 6-1). Under NaCl stress conditions, Fe deficiency caused a 54% decrease in Fe concentration in the roots at high UV-B radiation and a decrease of 53% at low UV-B radiation (Figure 6-1e; Table 6-1).

6.3.2.3.2 Salt effect, dependent on Fe and UV-B treatments

When Fe deficiency and NaCl stress conditions were jointly applied, they had no effect on Fe concentration in roots at high UV-B radiation but increased Fe concentrations in roots by 13% at low UV-B radiation (Figure 6-1e; Table 6-1). Under Fe-sufficient conditions, the addition of NaCl stress showed no change on the Fe concentration of roots.
grown at high UV-B radiation, but decreased Fe concentrations in roots by 30% in low UV-B radiation treatments (Figure 6-1e; Table 6-1).

6.3.2.3.3 **UV-B effect, dependent on Fe and salt treatments**

Under NaCl stress conditions, high UV-B applied showed no change of Fe concentration in roots under Fe-deficient and Fe-sufficient treatments (Figure 6-1e; Table 6-1). However, when there was no NaCl stress, additional UV-B radiation decreased Fe concentration in roots by 30% under Fe deficiency but had effect on Fe concentration in Fe-sufficient treatments (Figure 6-1e; Table 6-1).

6.4 **Discussion**

6.4.1 Main effects

6.4.1.1 Iron

In this study, Fe deficiency significantly decreased crude protein%, WSC%, Fe concentration in shoots and roots and increased the ADF% of wheat (Figure 6-1; Table 6-1). This is similar to the results reported by Bisht *et al.* (2002) and Yousfi *et al.* (2007) indicating a decrease in the protein% in leaves and Fe concentration in shoots and roots of Fe deficiency plants, respectively. Plant chloroplasts have high Fe requirements, because Fe activates a number of enzymes including the heme protein (cytochromes) Fe-sulphur (2Fe-2S) proteins ferredoxins (Bisht *et al.*, 2002). Additionally, decreases in protein under Fe deficiency may reflect lower enzyme levels that in turn decrease WSC% in plants. This study also found increased ADF% under Fe deficiency and this may indicate increased cellulose and lignin content, which in turn, could decrease digestibility (Bertrand *et al.*, 2008).
6.4.1.2 Salt

Salinity stress particularly decreased WSC%, and also crude protein% and ADF% in wheat plants (Figure 6-1a, b and c; Table 6-1), similar to the results reported by Glover et al. (2004) where NaCl decreased feed values in grasses. These authors suggest that NaCl stress decreased fibre content with increasing maturity and the decline in photosynthesis may induce decreased secondary cell wall deposition and lower fibre concentrations.

Additionally, Fe concentration increased in shoots, but decreased in roots under NaCl stress (Figure 6-1d and e; Table 6-1), similar to results reported by Rabhi et al. (2007) legumes. While NaCl stress also increased the Fe concentration in the shoots of lowland rice (Verma & Neue, 1984), NaCl stress decreased Fe concentration in the whole plant of barley and corn (Hassan et al., 1970). The results from the present study could suggest the NaCl-mediated translocation of Fe from roots to shoots, although Shiyab et al. (2003) suggested that high NaCl (> 100 mM NaCl) may decrease Fe transport in in vitro-grown shoots of sour orange. It must be pointed out however, that those authors used very high salinity levels (> 100mM NaCl), while in this study moderate salinity stress (75 mM NaCl) was used. Differences between experimental results may be attributed to differences in plant type and tissue, salinity concentration and composition, Fe concentration in the medium, growing conditions and the duration of study (Grattan & Grieve, 1999).

6.4.1.3 UV-B

In the previous chapter, it was shown that UV-B radiation can decrease root growth, but did not affect shoot growth (figure 4-3a and b; Table 4-1). The study further revealed decreases in Fe concentration in roots and increases in shoots under UV-B radiation (Figure 6-1d and e; Table 6-1), similar to results reported by Shukla & Kakkar (2002) in wheat. This is also similar to the findings from NaCl application and may indicate increased stress-induced Fe transport from the wheat roots to the shoots.
Chapter 6

6.4.2 Interaction effects

6.4.2.1 Fe x Salt

To the author’s knowledge there has been no research focusing on the interaction of Fe and NaCl stresses on crude protein% in plants. The current study showed that under NaCl stress, application of additional Fe deficiency decreased crude protein%, while this increased under the Fe sufficient treatment (Figure 6-1a; Table 6-1). It is known that Fe activates some enzymes involved in protein synthesis (Bisht et al., 2002) and NaCl stress can reduce secondary cell wall formation (Glover et al., 2004). This may help explain why crude protein% declines in under the combination of Fe deficiency and NaCl stress but increases under Fe-sufficient conditions. Additionally, when NaCl stress was applied, the additional Fe deficiency stress increased ADF% compared to Fe-sufficient treatments (Figure 6-1c; Table 6-1). However, the observed changes were very small (2-5%) and further studies, possibly with higher NaCl concentrations, need to be conducted to verify this finding.

When Fe deficiency and NaCl stress were applied together, an additional decrease in the Fe concentration in shoots and roots was found when compared with Fe deficiency alone (Figure 6-1d and e; Table 6-1). This is similar to the results reported by Rabhi et al. (2007) in Medicago, showing that NaCl stress may decrease Fe transport to shoots. However, the current study showed more PSs secretion under the combination of NaCl stress and Fe deficiency, compared Fe sufficient conditions (figure 5-2a; Table 5-2). More PSs were released under Fe deficiency when no NaCl stress was applied (-Fe x – NaCl), suggesting that Fe will be better absorbed under this condition compared to plants growing under the combination of Fe deficiency and NaCl stress (-Fe x +NaCl). This is supported by other findings of this study, showing Fe deficiency-induced decreases in root Fe levels that were less pronounced under no salinity stress, compared to the application of both stress factors (Figure 6-1e; Table 6-1).
6.4.2.2 Fe x UV-B

To the best of the author’s knowledge, there no research has thus for focussed on effects of the interaction of Fe stress with UV-B radiation for Fe content in shoots and roots. Under Fe deficiency, UV-B radiation decreased Fe concentration in roots (Figure 6-1d and e; Table 6-1) and this may be related to decreased PS release under UV-B radiation in wheat (figure 5-2a; Table 5-2).

6.4.2.3 Fe x Salt x UV-B

Thus far there has been no research focusing on three-way interactions between Fe, NaCl and UV-B radiation affect on Fe content in shoots and roots of plant. Under Fe deficiency conditions, the addition of NaCl and high UV-B radiation (-Fe x +NaCl x +UV-B) stress decreased the Fe concentration in shoots and roots more than in plants grown without NaCl and under low UV-B radiation (-Fe x –NaCl x –UV-B) treatments (Figure 6-1d and e; Table 6-1).

Under Fe deficiency conditions, the maintenance of Fe levels in shoots and roots is facilitated by PSs release (Figure 5-2a and b; Table 5-2). Additional NaCl and high UV-B radiation stress (-Fe x +NaCl x +UV-B) tended to reduce PSs release compared with under no NaCl and low UV-B radiation (-Fe x –NaCl x –UV-B) (Figure 5-2a and b; Table 5-2), thereby reducing Fe uptake by roots and resulting in decreased Fe levels in the plant.
6.5 Conclusions

The degree and direction of interactions among the stress factors applied here was dependent on the parameters examined. Crude protein%, WSC% and ADF% were decreased by Fe deficiency and salinity but were not affected by UV-B radiation, except that ADF% increased under Fe deficiency. With the exception of a significant Fe x NaCl interaction for protein% and ADF%, no other two-way or three-way interactions were observed for the stress factors applied. Additionally, WSC% did not change under any of the two-way or three interactions. This study shows that the Fe concentrations in shoots and roots reflect the results from PS release: as PSs release increases more Fe uptake by roots; therefore, directly increasing the Fe concentration in plants.
Chapter 7

7 General discussion and future directions

7.1 Introduction

These studies investigated various responses of wheat under the multiple stresses of Fe deficiency, salinity and UV-B radiation. There has been much research that focused on one stress or two-way interactions of these stress factors on plants, but most of them focussed on either aboveground or belowground effects. There has been a lack, however, of comprehensive investigations of multiple interactions of stress factors on plants, particularly on both aboveground and belowground responses. This thesis included the major stress factors Fe, NaCl and UV-B, including their two-way (Fe x NaCl, Fe x UV-B and NaCl x UV-B) and three-way interactions (Fe x NaCl x UV-B) on the morphological and physiological, biochemical and nutritive value responses of wheat plants. The overall objective of the thesis was to obtain an understanding of the interaction of these stress factors for wheat physiology.

7.2 General discussion

7.2.1 Major stresses

Global arable land area is increasingly exposed to one or more of the stress factors examined in this study. It is important to understand plant responses to these environmental stresses, especially for wheat plants because wheat is a domesticated grass that is cultivated worldwide. The results from this series of investigations can be drawn together in a conceptual model that suggests a fundamental relationship of plant responsiveness to these environmental stresses (Fe deficiency, salinity or UV-B radiation) (Figure 7-1). Under environmental stress plants, commonly decrease plant growth parameters but simultaneously increase biochemical protection. This in turn can contribute to the decreases in growth and feed value, due to the metabolic costs involved of a plant strategy for biochemical stress protection with lower allocation of carbon for productivity and feed value (Grime, 2001).
Figure 7-1. Model linking plant morphology and physiology, biochemical protection and plant nutritive value responses in wheat to environmental stresses such as Fe, NaCl and UV-B radiation. Chl = chlorophyll, PSs = phytosiderophores and WSC = water-soluble carbohydrates.
Based on the results of this study, a model can be explained to include the detailed responses in morphological and physiological attributes (Chapter 4), biochemistry (Chapter 5) and nutritive value (Chapter 6), as shown in Figure 7-2. Fe deficiency may cause decreases in plant morphological and physiological parameters, such as gas exchange and chlorophyll levels, while increases in the root:shoot ratio could suggest a plant response to optimise uptake of Fe from the soil (Zhang et al., 1991b). At the same time, wheat plants under Fe deficiency synthesise and release PSs to solubilise Fe in the rhizosphere (Römheld, 1987) and produce UV-absorbing compounds as a general stress response (Hunt & McNeil, 1998). Finally, morphological, physiological and biochemical responses under Fe deficiency may reduce plant nutritive value and increase fibre content, contributing to decreasing digestibility (Bertrand et al., 2008).

Plants grown under NaCl stress exhibited a decrease in several plant morphological and, physiological parameters and with slight increases in WUE. This might occur because of the large increases in biochemical protection, for example, proline in the NaCl treatment increased 16.3 fold compared to the no NaCl treatment. This suggested that proline can help osmotic adjustment and water status to maintain normal plant function under NaCl stress (Ashraf & Foolad, 2007) and it is supported by a lack of decline in photosynthetic function. It could also be argued that stress-induced proline accumulation in plants to such a high level requires energy and resources and thus contributes to reduced growth and lower production of sugars and protein levels, as exemplified here with reduced plant feed values such as crude protein%, WSC% and ADF%.

This study showed that although the UV-B radiation was applied aboveground it also affected plants belowground (for example, root DM and root:shoot). This may be because of increased investment of carbon for aboveground biochemical protection, for example, from UV-B-induced accumulation of UV-absorbing compounds (Figure 5-2; Table 5-2). The UV-B radiation tended to reduce release of PSs in wheat, suggesting that UV-B radiation might induce changes in gene expression. However, UV-B radiation had no affect on feed value, but increased Fe concentration in shoots and slightly decreased Fe concentration in roots. This suggested that UV-B radiation may induce Fe concentration transport from the wheat roots to the shoots.
Figure 7-2. Summary of the significant results of three environmental stress factors Fe, NaCl and UV-B radiation responses on plant morphological and physiological, chlorophyll, biochemical protection and nutritive value responses. Changes in plant attributes due to environmental stress are marked. Red font: increase and blue font: decrease. DM = dry matter, WUE = relative water content, Chl = chlorophyll, root:shoot = roots to shoots, PSs = phytosiderophores, WSC = water-soluble carbohydrates and ADF = acid detergent fibre.
7.2.2 Stress interaction

There has been some research on the effects of two-way interaction of the stress factors used here on plants, such as interactions between salinity and iron deficiency (Yousfi et al., 2007) and interaction between UV-B radiation and iron-deficiency (Zancan et al., 2008). However, to the best of the author’s knowledge, some of the stress response parameters investigated here under UV-B radiation, the interaction between UV-B radiation and NaCl stress (e.g. PSs release) and, particularly, the examination of the three-way Fe x NaCl x UV-B stress interaction, are investigated for the first time in this study. As discussed earlier, the two-way stress interaction can lead to increased (Dubé & Bornman, 1992) or decreased sensitivity (Hofmann et al., 2003; Murali & Teramura, 1985) of plants exposed to one stress to another stress.

7.2.2.1 Fe x NaCl

Shoot DM, root DM and leaf area were not accumulatively affected by the combination of Fe deficiency and NaCl stress. However, WUE increased under the interaction of Fe deficiency with NaCl stress but was not affected by Fe deficiency when there was no NaCl stress, suggesting that NaCl stress can alleviate Fe deficiency-driven decreases in WUE. This suggested that two-way stress will result in cross-tolerance causing mitigating stress effects (Hofmann et al., 2003). Under NaCl stress PSs release had decreased slightly under Fe deficiency compared to the no NaCl stress treatments. Yousfi et al. (2007) suggested that NaCl stress may inhibit PSs release induced by Fe deficiency because NaCl stress may increase root senescence and reduce the area of active root zones which, indirectly, can induce PSs synthesis and release in barley roots. When more PSs are released under Fe deficiency and no NaCl treatment, this is likely to result in higher Fe uptake and therefore lesser Fe deficiency-generated reduction of Fe levels in shoots and roots (as observed here, together with higher crude protein levels), compared to Fe deficiency application in combination with NaCl. Simultaneously, the ADF% had increased more under NaCl stress and additional Fe deficiency compared to Fe sufficient treatments, suggesting that this co-occurring stress might induce decreased secondary cell wall deposition and lower fibre concentrations (Glover et al., 2004).
7.2.2.2 Fe x UV-B

Generally, the interaction between Fe deficiency and UV-B radiation had no effect on plant morphology and physiology and gas exchange, except for possible stomatal conductance decreases under combined Fe deficiency and UV-B radiation. There is a general lack of information about plant biochemical protection and plant morphological and physiological effects under this stress combination. This study reported that UV-absorbing compounds were synergistically enhanced in the combination of UV-B radiation and Fe deficiency stress. Logemann and Hahlbrock (2002) suggested that common stress regulatory factors may elicit a synergistic effect. Iron concentration in shoots and roots were additionally decreased under UV-B radiation and Fe deficiency compared with the Fe sufficient treatments. This could suggest that the cost of increased UV-absorbing compound accumulation under this two-way interaction resulted in decreased Fe concentration in the shoots and roots. Iron concentration in shoots and roots decreased more under high UV-B radiation compared to low UV-B radiation treatments and high UV-B radiation also decreased PSs release. Thus Fe uptake would be more affected by UV-B radiation compared with low UV-B radiation treatments.

7.2.2.3 NaCl x UV-B

This study reported that the interaction between NaCl stress and UV-B radiation had no significant interaction effect on wheat plants contradicting findings by Çarkırlar et al. (2008) who showed NaCl induced cross-acclimation to UV-B radiation in four barley (Hordeum vulgare L.) cultivars. This discrepancy may be due to the use of a different cultivar and lower levels of salinity stress in the present study.
7.2.2.4 Fe x NaCl x UV-B

Overall, this study found few three-way interaction effects on plants, PS release increased more under Fe deficiency conditions alone (-Fe x –NaCl x –UV-B) than when Fe deficiency was applied with NaCl and high UV-B radiation (-Fe x +NaCl x +UV-B). It is suggested that NaCl increased root senescence and reduced active root zones (Yousfi et al., 2007) and high UV-B radiation treatments tended to decrease amino acids (Moorthy & Kathiresan, 1998), both of which would reduce PS release. Additionally, this study showed that the cumulative application of the three stress factors reduced Fe concentration in roots and this may either reflect lower Fe uptake or increased translocation from roots to shoots.
7.3 Conclusions

In summary, the main findings of this study are as follows.

1. Iron deficiency and NaCl stress generally decreased plant morphological and physiological attributes as well as nutritive value, while increasing biochemical protection in the form of proline accumulation and resource acquisition in the form of PS release.

2. Ultraviolet-B radiation increased UV-absorbing compound levels in leaves but also affected belowground physiological attributes, such as reduced root DM and decreased Fe concentration in roots, together with reduced PS release.

3. The interaction between Fe deficiency and NaCl stress showed an accumulative effect on plant nutrient acquisition in the form of slightly decreased PS release.

4. Iron deficiency in combination with UV-B radiation treatments showed a synergistic effect on UV-absorbing compounds and an accumulative effect by decreasing Fe concentration in shoots and roots.

5. The application of salinity stress in combination with UV-B radiation showed few interaction effects.

6. The three-way interaction (Fe x NaCl x UV-B) slightly decreased PS release and had a cumulative effect by decreasing Fe concentration in roots.
7.4 Future directions

Although previous studies have been carried out on the individual effects of the three stress factors, Fe, NaCl and UV-B on wheat plants, there is still a poor understanding of the interplay between the physical environment and the effects of these three stress factors on naturally growing plants. Results from this thesis suggest that further investigations are needed in the following dissections.

- Investigations are needed of multiple interactions between these three stress factors (Fe, NaCl and UV-B) on other varieties of plants.

- This should also include the study of the effects on plants of different levels within these three stress factors.

- The differences in UV-absorbing compound accumulation could be studied more closely, for example by examining flavonoids to confirm the effective pigments involved in this study.

- Studies following a more extended timeframe than used here could provide more information on whether these stress interactions could lead to long-term responses in the parameters studied.

- Increased PSs release measurements every two hours would provide more information on how the diurnal pattern of PSs release is mediated by the stress factors involved.

- Other examinations would include the effects of spectral quality, e.g. of different wavelength bands within the PAR and UV regions on PS release and Fe uptake in grasses.
• An important further extension would be to compare the results of the current study with those in the field because results of controlled experimental studies may differ from those in the natural world.

• Further, future work could extend the study by looking at seed yield and reproductive growth, i.e. factors which are directly important to wheat growers.

### 7.5 Practical implications

While this research project was conducted under controlled environmental conditions, the findings indicate the following practical implications for wheat growers. Future field studies would further substantiate these suggestions.

• When wheat is grown in saline soils, the provision of fertiliser containing sufficient levels of Fe is needed to ensure adequate physiological and biochemical plant function, as well as nutritive value in the wheat produced, including increased PS release and, as a consequence, increased levels of Fe in shoots and roots, as well as higher protein and lower fibre content.

• Under high UV-B radiation conditions, Fe-containing fertilizer needs to be applied to ensure adequate Fe levels in shoots and roots in wheat.
8 References


Appendices

Appendix 1. Morphological and physiological attributes in wheat plants (mean ± standard error, n=6 except n=3 for RWC), grown under three stress treatments and their interactions: Fe, NaCl and UV-B radiation. DM, dry weight; WUE, water use efficiency. Analysis of WUE data was square root-transformed data.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot DM (g plant⁻¹)</th>
<th>Root DM (g plant⁻¹)</th>
<th>Root:shoot</th>
<th>Leaf area (cm² plant⁻¹)</th>
<th>RWC (%)</th>
<th>Photosynthesis (µmol CO₂ m⁻² s⁻¹)</th>
<th>Conductance (mol H₂O m⁻² s⁻¹)</th>
<th>Transpiration (mmol H₂O m⁻² s⁻¹)</th>
<th>WUE (mmol CO₂ mol⁻¹H₂O)</th>
</tr>
</thead>
</table>
| -NaCl -UV-B | +Fe 0.31 ± 0.009 0.10 ± 0.004 0.31 ± 0.011 20.7 ± 7.70 92.9 ± 1.07 18.8 ± 1.14 0.71 ± 0.102 5.96 ± 0.481 3.18 ± 0.430  
                       -Fe 0.10 ± 0.010 0.04 ± 0.003 0.36 ± 0.015 6.33 ± 4.011 94.3 ± 1.83 4.94 ± 2.707 0.53 ± 0.092 4.94 ± 0.623 0.62 ± 0.190 |
| -NaCl +UV-B | +Fe 0.30 ± 0.014 0.09 ± 0.003 0.23 ± 0.009 22.8 ± 7.66 93.6 ± 0.94 20.0 ± 21.01 1.00 ± 0.161 7.23 ± 0.871 2.81 ± 0.404  
                       -Fe 0.08 ± 0.007 0.03 ± 0.002 0.35 ± 0.011 6.63 ± 3.877 95.7 ± 0.42 6.30 ± 2.260 0.70 ± 0.144 6.54 ± 1.092 0.78 ± 0.213 |
| +NaCl -UV-B | +Fe 0.14 ± 0.007 0.06 ± 0.003 0.42 ± 0.007 1.88 ± 0.644 90.2 ± 0.33 20.3 ± 1.26 0.60 ± 0.101 5.64 ± 0.804 3.95 ± 0.480  
                       -Fe 0.06 ± 0.002 0.03 ± 0.002 0.49 ± 0.012 1.02 ± 0.422 89.7 ± 4.93 9.95 ± 2.014 0.55 ± 0.101 5.46 ± 0.785 1.84 ± 0.333 |
| +NaCl +UV-B | +Fe 0.13 ± 0.006 0.05 ± 0.001 0.41 ± 0.013 1.02 ± 0.444 91.3 ± 1.26 23.1 ± 1.24 1.00 ± 0.072 8.20 ± 0.401 2.82 ± 0.405  
                       -Fe 0.06 ± 0.002 0.03 ± 0.001 0.45 ± 0.016 0.41 ± 0.163 86.5 ± 3.71 6.48 ± 1.040 0.46 ± 0.133 4.48 ± 0.955 1.61 ± 0.311 |
Appendix 2. Physiological attributes in wheat plants (mean ± standard error, n=6), grown under three stress treatments and their interactions: Fe, NaCl and UV-B radiation. Chl, chlorophyll; UV Abs, UV absorbing compounds; PSs, phytosiderophores; Chl b analyses were based on square root transformed data.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chl total (mg g⁻¹ DM)</th>
<th>Chl a (mg g⁻¹ DM)</th>
<th>Chl b (mg g⁻¹ DM)</th>
<th>Chl a:b</th>
<th>UV-absorbing compounds (mg DM ml⁻¹)</th>
<th>PSs (µmol g⁻¹ root DW)</th>
<th>Proline (µg mg⁻¹ DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-NaCl +Fe</td>
<td>14.46 ± 0.46</td>
<td>10.39 ± 0.26</td>
<td>4.50 ± 0.12</td>
<td>2.31 ± 0.04</td>
<td>1.28 ± 0.04</td>
<td>3.73 ± 1.33</td>
<td>1.80 ± 0.24</td>
</tr>
<tr>
<td>-UV-B -Fe</td>
<td>14.89 ± 0.35</td>
<td>3.79 ± 0.40</td>
<td>2.12 ± 0.08</td>
<td>1.76 ± 0.09</td>
<td>1.30 ± 0.06</td>
<td>11.73 ± 2.61</td>
<td>1.94 ± 0.21</td>
</tr>
<tr>
<td>-NaCl +Fe</td>
<td>12.56 ± 0.36</td>
<td>10.03 ± 0.33</td>
<td>4.43 ± 0.11</td>
<td>2.26 ± 0.03</td>
<td>1.27 ± 0.03</td>
<td>0.98 ± 0.59</td>
<td>1.76 ± 0.24</td>
</tr>
<tr>
<td>+UV-B -Fe</td>
<td>13.45 ± 0.26</td>
<td>3.81 ± 0.21</td>
<td>2.09 ± 0.08</td>
<td>1.82 ± 0.07</td>
<td>1.24 ± 0.07</td>
<td>5.25 ± 0.45</td>
<td>2.17 ± 0.35</td>
</tr>
<tr>
<td>+NaCl +Fe</td>
<td>5.89 ± 0.26</td>
<td>9.31 ± 0.16</td>
<td>4.17 ± 0.11</td>
<td>2.24 ± 0.05</td>
<td>1.67 ± 0.05</td>
<td>6.12 ± 0.79</td>
<td>28.52 ± 3.25</td>
</tr>
<tr>
<td>-UV-B -Fe</td>
<td>5.92 ± 0.51</td>
<td>3.02 ± 0.23</td>
<td>1.87 ± 0.07</td>
<td>1.60 ± 0.08</td>
<td>1.92 ± 0.05</td>
<td>8.56 ± 2.45</td>
<td>29.23 ± 2.95</td>
</tr>
<tr>
<td>+NaCl +Fe</td>
<td>4.87 ± 0.19</td>
<td>8.70 ± 0.25</td>
<td>3.85 ± 0.11</td>
<td>2.26 ± 0.05</td>
<td>1.69 ± 0.05</td>
<td>2.66 ± 0.23</td>
<td>34.59 ± 1.48</td>
</tr>
<tr>
<td>+UV-B -Fe</td>
<td>4.90 ± 0.28</td>
<td>2.98 ± 0.14</td>
<td>1.90 ± 0.07</td>
<td>1.57 ± 0.07</td>
<td>1.76 ± 0.07</td>
<td>6.87 ± 0.77</td>
<td>32.46 ± 4.04</td>
</tr>
</tbody>
</table>
Appendix 3. Physiological attributes in wheat plants (mean ± standard error, n=6), grown under three stress treatments and their interactions: Fe, NaCl and UV-B radiation. WSC, water-soluble carbohydrate; and ADF, acid detergent fibre.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cure protein (%)</th>
<th>WSC (%)</th>
<th>ADF (%)</th>
<th>Fe in shoot (µg g⁻¹ DM)</th>
<th>Fe in root (µg g⁻¹ DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-NaCl -UV-B</td>
<td>+Fe 25.74 ± 0.61</td>
<td>11.79 ± 0.58</td>
<td>23.77 ± 0.39</td>
<td>56.81 ± 6.49</td>
<td>54.85 ± 1.96</td>
</tr>
<tr>
<td></td>
<td>-Fe 24.81 ± 0.41</td>
<td>8.68 ± 0.41</td>
<td>24.62 ± 0.14</td>
<td>41.02 ± 8.15</td>
<td>41.31 ± 3.71</td>
</tr>
<tr>
<td>-NaCl +UV-B</td>
<td>+Fe 25.34 ± 0.77</td>
<td>11.70 ± 0.48</td>
<td>23.40 ± 0.16</td>
<td>75.39 ± 3.27</td>
<td>59.45 ± 2.25</td>
</tr>
<tr>
<td></td>
<td>-Fe 26.05 ± 0.85</td>
<td>8.60 ± 0.40</td>
<td>24.16 ± 0.13</td>
<td>36.26 ± 1.40</td>
<td>30.66 ± 1.93</td>
</tr>
<tr>
<td>+NaCl -UV-B</td>
<td>+Fe 27.36 ± 0.56</td>
<td>7.84 ± 0.32</td>
<td>22.84 ± 0.11</td>
<td>84.80 ± 3.74</td>
<td>61.73 ± 2.08</td>
</tr>
<tr>
<td></td>
<td>-Fe 20.45 ± 0.87</td>
<td>3.04 ± 0.55</td>
<td>24.24 ± 0.22</td>
<td>35.48 ± 1.46</td>
<td>29.07 ± 1.02</td>
</tr>
<tr>
<td>+NaCl +UV-B</td>
<td>+Fe 27.44 ± 0.68</td>
<td>6.63 ± 0.46</td>
<td>22.57 ± 0.14</td>
<td>92.96 ± 4.59</td>
<td>57.37 ± 1.26</td>
</tr>
<tr>
<td></td>
<td>-Fe 21.23 ± 1.09</td>
<td>3.71 ± 0.60</td>
<td>24.15 ± 0.33</td>
<td>39.79 ± 1.10</td>
<td>26.28 ± 0.34</td>
</tr>
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</table>
Appendix 4. The attached CD contains the statistical output of REML analyses in GenStat.